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13. Abstract (Maximum 200 Words) <i>(abstract should contain no proprietary or confidential information)</i> Patients with breast cancers that express estrogen receptor (ER) commonly receive antiestrogen therapy. The efficacy of this therapy depends on close regulation of breast growth by estrogen. However, as breast cancers progress, they often become resistant to estrogens, and most patients no longer respond to antiestrogen therapy. New antiestrogen treatment options are needed, and alternative therapies may derive from findings showing that some ER molecules occur in plasma membranes of breast cancer cells and interact with transmembrane HER-2 growth factor receptors. Expression of HER-2 receptors occurs in many breast cancers, and the protein kinase activity of HER-2 may modulate ligand-independent activation of ER. If active cross-communication between ER and HER-2 receptor occurs and leads to the promotion of cancer growth, this axis may offer a new target for therapeutic intervention. We have detected a membrane-associated form of ER in breast cancer cells and have evidence that it promotes tumor growth. Using this novel signaling pathway as a target, we are now testing new treatment options to prevent cancer progression in models of human breast cancer. Since HER-2 overexpression in breast cancer is associated with failure of antiestrogen therapy, understanding the basis of associations between ER and HER-2 receptors may help to improve patient management and enhance survival.				
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INTRODUCTION

Breast cancer is the most common malignancy in women in North America and is usually a disease of post-menopausal women (1). In the clinic, endocrine therapy is an important intervention in women with breast cancers that express the estrogen receptor (ER). Treatment with tamoxifen and other antiestrogens has enhanced the survival of breast cancer patients, and these agents are now used in breast cancer prevention. The success of endocrine therapy in breast cancer is dependent on the close regulation of breast cell growth by steroid hormone receptors (1,2). However, as this cancer progresses, it usually becomes resistant to estrogens, and most patients no longer respond to therapy with tamoxifen or other antiestrogens. New information on the existence of an alternate estrogen signaling pathway in breast cancer cells may promote the design of novel and more effective antihormone treatments for human breast cancers (3).

Growth factor receptor malfunction also occurs in malignant progression, with members of the HER-1 (EGF) family frequently implicated in human cancer (1-3, 4-8). The HER (erb B) receptor family includes the HER-2 (erb B2) protein, a 185-kD transmembrane tyrosine kinase encoded by HER-2 oncogene (9-11), the HER-3 protein (12) and HER-4 receptor (13,14). Overexpression of HER-2 or related growth factor receptors is estimated to occur in two-thirds of sporadic breast cancers (1), while HER-2 amplification or overexpression is found in 25-30% of breast cancers in women and 41% of breast cancers in men (15-18). Overexpression of HER-2 is a marker of poor prognosis (15-19) and is associated with failure of antiestrogen therapy (3,20-31).

Receptors for estrogen occur in a family of potentially oncogenic receptors. Sequence similarities between the erb A gene product of avian erythroblastosis virus and ER suggest that these two proteins likely evolved from a common gene (32). Erb A genes cannot induce cell transformation alone, but cooperate with the viral erb B oncogenes in cell transformation (33). With this lineage of cooperativity between erb A and erb B genes, it is not surprising to find reports of significant cross-talk and interaction between erb B (HER) pathways and estrogen receptor signaling (3,24,27,34-36).

It is generally held that the biologic activity of estrogen in the breast is mediated through the specific high-affinity ER located in breast cell nuclei (1,37). In the absence of estrogen, ER is considered to associate with proteins that prevent its interaction with the cell transcription apparatus. Upon estrogen binding, the receptor undergoes an activating conformational change that promotes association with target genes, thus permitting regulation of gene transcription [see FIG. 1]. In addition to the latter pathway, however, estrogen can also induce extremely rapid increases in the levels of intracellular second messengers, including calcium (39,40) and cAMP (41,42), as well as activation of MAP kinase (43,44) and phospholipase (45). The timecourse of these events is similar to those elicited by peptides, lending support to the hypothesis that they do not involve the classical genomic action of estrogen. Both estrogens and growth factor ligands act as mitogens to promote cell growth in the breast, and the cellular effects of these agents sometimes overlap. The molecular details of this cross-talk between ER and erb B receptors are now beginning to emerge, and ER itself may be an important point of convergence (3,24,34-36).

Many of the rapid effects of estrogen are now attributed to the action of the hormone at the cell membrane, and these biologic actions appear to be mediated by membrane receptors that bind estrogen. The isolation and structural characterization of these native macromolecules have not yet been accomplished, and the derivation and functions of this receptor (or receptors) are largely unknown. Since activation of this alternate signaling pathway by estrogens may represent a mechanism by which estrogens regulate proliferation, we have investigated the nature and activity of this membrane response pathway in human breast cancer cells. Classical models of estrogen action that characterize this signaling pathway as solely due to the activity of an intracellular ligand-dependent transcription factor are clearly incomplete and must be modified to include estrogen receptors as significant components of other signaling pathways. As urged by others (40), "these data beg a reevaluation of the relative contributions of genomic and nongenomic activities in ER biology, an activity that is likely to support the development of pharmaceutical agents that exert differential activities in the two pathways".

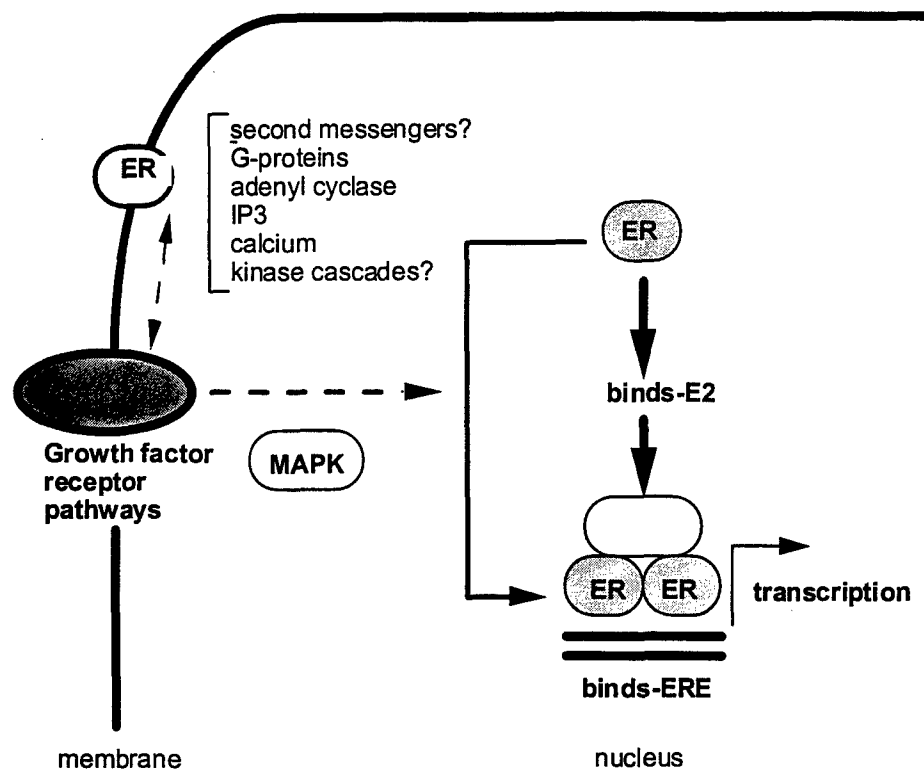


FIG. 1. Postulated cellular mechanism of action of estrogen (E2) and growth factors in breast cancers with estrogen receptor (ER). In most models of estrogen action, estrogen binding to ER in the nucleus promotes receptor dimer formation and receptor phosphorylation that enhances binding to nuclear estrogen-responsive elements (ERE) and coactivator proteins, leading, in turn, to initiation of gene transcription. However, this model fails to account for numerous, rapid cell responses to estrogen (41-69). In the hypothesis to be tested here, estrogen may also bind to a membrane ER, with potential for stimulation of estrogenic responses via an alternate pathway. Current reports suggest that membrane-associated ER may activate one or more pathways, including interaction with growth factor membrane receptors such as HER-2 or activation of G-proteins, adenylate cyclase, inositol phosphate, calcium homeostasis and/or MAP kinase. These interactions may promote phosphorylation of ER via estrogen-induced activation of second-messengers and protein kinases or, alternatively, via ligand-independent pathways involving growth factor receptors. Growth of cells treated with estrogen may occur as a consequence of a synergistic feed-forward circuit where estrogen activates membrane signaling pathways that act, in turn, to enhance transcriptional activity of ER in the nucleus.

RESEARCH PROGRESS

Aim 1) To assess the existence and identity of receptors for estrogen in plasma membranes of human breast cancer cells.

1.a. Enrichment of high-affinity binding-sites with specificity for $E_2\beta$ in breast cancer cell plasma membranes

To confirm earlier reports of membrane binding-sites for $E_2\beta$ (52,55,61,63), we measured specific [3H]E $_2\beta$ binding in subcellular fractions of MCF-7 cells after controlled cell homogenization and fractionation (47,48). With recovery of more than 97% of total $E_2\beta$ binding found in homogenates of MCF-7 cells, specific [3H]E $_2\beta$ binding was distributed among crude nuclear, microsomal, mitochondria-lysosome and cytosol fractions (see Fig. 1 in ref. 84). After purification of plasma membranes from the crude nuclear fraction by use of discontinuous-sucrose density gradient centrifugation, the PM fraction showed enhanced activity of 5'-nucleotidase, a plasma membrane marker enzyme, to about 23-times that of homogenate. Specific [3H]E $_2\beta$ binding in plasma membranes was enriched to 28-times homogenate activity and represented 22% of homogenate binding. This data shows that specific $E_2\beta$ binding co-purifies with a plasma membrane marker

protein in membrane fractions from breast cancer cells. LDH activity, highly enriched in cytosol, is not significantly detected in PM (84). In addition, cell DNA recovery was 94 ± 3 % of homogenate levels in nuclear fractions, and no DNA was detected in PM fractions (84).

Binding of [3 H]E $_2$ β by PM fractions from MCF-7 cells was analyzed further in equilibrium binding studies (see Figs. 2,3 in ref. 84). Samples of PM were exposed to [3 H]E $_2$ β concentrations ranging from 1×10^{-10} M to 5×10^{-9} M. Binding of hormone by PM is saturable, and Scatchard analyses of specific [3 H]E $_2$ β binding (cf. 48) show that the dissociation constant for the binding process is 3.6×10^{-10} M. Total binding sites in PM at saturation correspond to approximately 6.7 pmol E $_2$ β per mg membrane protein (84). In comparison with the estradiol binding properties of intact MCF-7 cells, plasma membrane estrogen-binding sites retain high affinity for specific estradiol binding and exhibit significant enrichment of ligand-binding capacity (see ref. 3). Further, ligand specificity of [3 H]E $_2$ β binding to PM was established by effective suppression by a 100-fold molar excess of unlabeled E $_2$ β (84). In contrast, [3 H]E $_2$ β binding by PM was essentially uninfluenced by these levels of estradiol-17 α , progesterone or testosterone (84).

1.b. Identification of estrogen receptor forms in subcellular fractions after gel electrophoresis

To characterize putative estrogen receptor forms associated with PM fractions, samples were subjected to Western blot analysis, and blots were probed either with anti-ER antibody Ab2 or with E $_2$ β -POD (84). PM purified from MCF-7 cells show significant enrichment of a primary 67-kDa protein that reacts strongly with antibody Ab2 to LBD of nuclear ER- α (see Fig. 4 in ref. 84). Similarly, breast cell nuclear fractions are enriched with this protein reactive with ER- α (84). The 67-kDa band also shows evidence of specific labeling with E $_2$ β -POD (84). A secondary band at 46-kDa and minor bands at 62-kDa and 97-kDa were detected in PM and other cell fractions by use of Western blot and ligand-blotting (84). Using an antibody directed to ER- β , no significant reactivity with proteins at the expected size of 58-62 kDa was found in homogenate, nuclear or plasma membrane fractions of the MCF-7 cells (84).

1.c. Purification of candidate receptors

As indicated above, work aimed at purification of candidate receptors has begun. As outlined in the original proposal, our initial efforts have involved the use of affinity chromatography, with recovered receptor to be used for preparation of monoclonal antibodies and for further molecular characterization and functional studies using cDNA for membrane ER. However, the yield of estrogen-binding receptor protein from purified plasma membranes has been limited, and this strategy may require some modification as suggested from recent studies on the isolation of membrane-associated receptors for progestins (85). Our efforts to isolate membrane-associated estrogen receptors from caveola-related subfractions of breast cancer cell plasma membranes will be reported at the upcoming Era of Hope Meeting, 2002 (86).

Aim 2) To assess the role of membrane estrogen receptors in promoting growth of breast cancers.

2.a. Rapid effects of E $_2$ β and E $_2$ β -BSA on activation of MAPK and Akt kinase in breast cancer cells

Post-receptor signal transduction events, such as stimulation of MAPK, extracellular signal-regulated kinase ERK-1 (p44) and ERK-2 (p42) (43,61), may contribute to proliferative effects of E $_2$ β in breast cells. Thus, we assessed estrogen-induced phosphorylation of MAPK in MCF-7 cells *in vitro*. E $_2$ β , but not 17 α -estradiol (E $_2$ α), promotes phosphorylation of MAPK isoforms, with effects evident within 2 min (see Fig. 5 in ref. 84). To test whether activation of MAPK by E $_2$ β may be mediated by binding of estrogen to membrane-associated receptors, MCF-7 cells were treated with E $_2$ β linked to BSA, a macromolecular complex considered to be membrane-impermeant (52,61). Using E $_2$ β -BSA, but not control E $_2$ α -BSA, phosphorylation of MAPK isoforms is again evident within 2 min of steroid administration. Incubation of cells with antibody against LBD of ER (Ab2) inhibited MAP kinase phosphorylation induced by E $_2$ β or E $_2$ β -BSA. Similarly, we assessed signaling via the phosphatidylinositol-3 kinase (PI3K)/Akt pathway after treatment of MCF-7 cells with E $_2$ β or

E₂β-BSA. Both ligands induced significant activation of Akt kinase (84), and inhibition of estrogen-induced effects occurred when cells were preincubated with ER antibody (Ab2), pure antiestrogen (ICI 182,780) or the PI3K inhibitor, LY 294002.

To assess the potential for MCF-7 cell activation by free estradiol liberated from E₂β-BSA, we transfected MCF-7 cells with an ERE-CAT reporter gene as before (3). Cells were exposed *in vitro* to free estradiol-17β or to E₂β-BSA for only 10 minutes, then washed and incubated further. After 24 hours, ERE-CAT reporter gene activity was measured. Short-term treatment with free estradiol-17β stimulated a marked increase in reporter gene activity ($P < 0.001$), but E₂β-BSA elicited no significant effect (see Fig. 6 in ref. 84).

Since interaction of E₂β-BSA with plasma membrane binding-sites may be required for intracellular signaling (52,61), we evaluated binding of fluorescein-labeled E₂β-BSA (E₂β-BSA-FITC) in MCF-7 cells. E₂β-BSA-FITC binds at the surface of 77% of MCF-7 cells (see Fig. 7 in ref. 84), while only minimal background fluorescence is found among cells incubated with control ligand, BSA-FITC (84). In additional control studies, ER-positive ZR-75 breast cancer cells, as MCF-7 cells, show retention of E₂β-BSA-FITC at the cell surface, but ER-negative MDA-MB-231 breast cancer cells or COS-7 cells do not show significant binding of E₂β-BSA-FITC at the external membrane (84). On flow cytometric analysis (84), the E₂β-BSA-FITC complex shows evidence of ligand specificity, with significant reduction ($P < 0.01$) of E₂β-BSA-FITC binding by competition with equi-molar amounts of free E₂β, E₂β-BSA, tamoxifen or ICI 182, 780, while the related steroid congener, progesterone, is not effective. Surface binding of E₂β-BSA-FITC is also significantly diminished by competition with antibody to the LBD of nuclear ER, suggesting some immunologic identity of the membrane site with nuclear ER (84). As expected, after permeabilization of cells by disruption of plasma membrane with detergent, intense labeling of ER in cell nuclei is found and occurs in 96% of breast cancer cells (84). In other control studies, MDA-MB-231 cells with no ER showed no binding or retention of E₂β-BSA-FITC label, while ZR-75 breast cancer cells with ER expression did show surface binding of the complex (84).

2.b. Inhibition of cell growth in vitro by antibody to ligand-binding domain of ER-α

Since antibodies to cell surface growth factor receptors are sometimes effective in blocking tumor growth (3,77), antiproliferative activity of antibodies to ER-α was evaluated using MCF-7 cells *in vitro*. The estrogen-dependent MCF-7 cells show enhanced growth after treatment with E₂β, but not E₂α (see Fig. 8 in ref. 84). However, prior exposure to LBD Ab1 or LBD Ab2 elicits a significant reduction ($P < 0.05$) in the E₂β growth response (84).

Since some recent studies suggest that the proliferative response to E₂β is committed within 1 min and is evoked by activation of only a small fraction ($\leq 5\%$) of ER (73), we assessed the growth of breast cells after brief treatment with E₂β-BSA. MCF-7 cells were treated with 0.5 μM E₂β-BSA for only 10 min. Then, cells were rinsed and cultivated in estrogen-free media for an additional 72 h. The results show that E₂β-BSA ($P < 0.001$), but not control E₂α-BSA, stimulates cell growth (84). Moreover, the proliferative effect of E₂β-BSA is blocked by treatment of cells with ICI 182,780, a pure antiestrogen ($P < 0.001$) (84), or by prior exposure to anti-ER Ab1 ($P < 0.05$) or Ab2 ($P < 0.001$) (84).

Aim 3) To investigate new treatment options to prevent breast cancer progression in human breast cancer.

3.a. Inhibition of breast tumorigenesis in vivo by antibody to ligand-binding domain of ER-α

The antitumor activity of antibodies to ER-α was evaluated further using MCF-7 tumors *in vivo*. MCF-7 cells were grown as subcutaneous xenografts in female athymic mice primed with E₂β to promote growth of these estrogen-dependent cells (3). Antibody or control treatments were initiated when tumors grew to $> 30 \text{ mm}^3$. Anti-ER Ab2 was administered in 6 doses over a 26-day period. The results show that antibody to ER, but not control immunoglobulin, elicits a significant suppression of tumorigenesis of human MCF-7 breast cancer xenografts in female nude mice treated concomitantly with E₂β (see Fig. 8 in ref. 84).

3.b. Estrogen receptor interactions with growth factor membrane receptors

As noted above, activation of estrogen receptor- α (ER) by growth factors in the absence of estrogen is a well-documented phenomenon. To further study this process of ligand-independent receptor activation, COS-7 cells without ER were transfected with both ER and epidermal growth factor (EGF) receptor. In the absence of estrogen, EGF stimulated rapid tyrosine phosphorylation of ER in transfected COS-7 cells (see ref. 87). Similarly, in MCF-7 breast cancer cells that have natural expression of ER and EGF receptors, EGF promoted acute phosphorylation of serine and tyrosine residues in ER, and a direct interaction between ER and EGFR after treatment with EGF was found (87). In confirmation of a direct interaction between ER and EGF receptors, activation of affinity-purified EGF receptor tyrosine kinase *in vitro* stimulated the phosphorylation of recombinant ER (87). The cross-communication between EGFR and ER appears to promote significant stimulation of cell proliferation and a reduction in the apoptotic loss of those cells that express both receptor signaling pathways (87). However, COS-7 cells transfected with both ER and EGF receptors show minimal stimulation of classical estrogen response element (ERE)-dependent transcriptional activity after stimulation by EGF ligand. This suggests that the proliferative and antiapoptotic activity of EGF-induced ER activation may be dissociated from ERE-dependent transcriptional activity of the estrogen receptor. Further consideration of the cross-communication between membrane-associated ER and membrane growth factor receptors, such as EGF and HER-2 receptors, may provide new targets for intervention in the clinic (88).

KEY RESEARCH ACCOMPLISHMENTS

- Plasma membrane-associated binding sites with high affinity and specificity for estradiol-17 β occur in human breast cancer cells.
- Plasma membrane-associated binding sites for estradiol in human breast cancer cells may play a role in modulating cell growth and survival.
- Plasma membrane-associated estrogen receptors interact with signaling initiated by membrane growth factor receptors

REPORTABLE OUTCOMES

Presentations

1. "Interactions between Type I receptor tyrosine kinases and steroid hormone receptors : Therapeutic implications". Presented at *First International Symposium on Translational Research in Oncology*, Dublin, Ireland (2001).
2. "HER-2 receptor signaling modulates estrogen receptor in breast cancer". Presented at Medical Oncology Seminar Series, UCLA School of Medicine (2001).
3. "Steroid and growth factor receptors: Cross-talk and clinical implications". Presented at *Second International Symposium on Translational Research in Oncology*, Anaheim, California (2002).

Abstracts

1. Marquez, D.C., Chen, H.-W. and Pietras, R.J. (2002). Membrane-associated estrogen receptors localize to caveola-related domains and contribute to growth regulation of breast cancer cells. DOD Breast Cancer Research Program Era of Hope Meeting Proceedings (in press).

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1. Pietras, R.J., Nemere, I. and Szego, C.M. (2001). Steroid hormone receptors in target cell membranes. Endocrine 14 : 417-427.
2. Marquez, D.C. and Pietras, R.J. (2001). Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. Oncogene 20: 5420-5430.
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5. Marquez, D.C. and Pietras, R.J. (2002). Membrane-associated estrogen receptors and breast cancer. In: Identities of Membrane Steroid Receptors (Watson, C., editor), Kluwer Academic Publishers (in press).
6. Pietras, R.J. (2002). Steroid and growth factor receptors: Cross-talk and clinical implications. In: Breast Cancer Management, 2nd Edition (Reese, D., Nabholz, J.-M., and Slamon, D.J., editors), Lippincott, Philadelphia (in press).

No abstracts, patents, degrees, development of cell lines, informatics or additional funding or research opportunities to be reported at this time.

CONCLUSIONS

A new approach to cancer therapy involves efforts to cut the lines of communication between hormone receptors and the cell nucleus, thus slowing or blocking cell division. Antiestrogen therapy is one well-known example of this approach, and it is often used to treat breast cancer and to prevent recurrence. Unfortunately, many patients do not respond to current therapy, and almost all treated patients eventually become resistant to antiestrogens. In addition, antiestrogens that are now available can result in abnormal uterine growth and thromboembolic events. The failure of antihormone therapy in the clinic is due to many factors, including the emergence of estrogen-independent growth that is no longer responsive to treatment with antiestrogen agonists.

New options for antiestrogen treatment are clearly needed, and alternative therapies may now derive from the current findings showing that ER molecules occur not only in the nucleus of the cell, but also in association with the surface membranes of human breast cancer cells. Moreover, these ER may interact with membrane HER-2 growth factor receptors. It is known that expression of HER-2 receptors occurs in many human breast cancers, and the enzyme activity of HER-2 may play a role in ER activation even in the absence of estrogen. If active cross-talk between ER and the HER-2 growth factor receptor occurs and leads to promotion of cancer growth, this signaling axis may offer a new target for therapeutic intervention. Since overexpression of HER-2 in human breast cancers is associated with the failure of antiestrogen therapy in the clinic, understanding the biologic basis of the association between membrane ER and HER-2 receptors may help to improve decisions on patient management and to increase patient survival.

In the present work, we have made good progress in ascertaining the existence and nature of receptors for estrogen in surface membranes of human breast cancer cells. We have begun to assess the role of membrane ER in promoting growth of breast cancers. In challenging the dogma of estrogen action exclusively via an intracellular receptor, this work may lead to the development of previously unsuspected, less toxic antitumor therapies targeted to human breast cancer cells.

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**MEMBRANE -ASSOCIATED ESTROGEN
RECEPTORS LOCALIZE TO CAVEOLA-RELATED
DOMAINS AND CONTRIBUTE TO GROWTH
REGULATION OF BREAST CANCER CELLS**

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Estrogen receptors (ER) are present in about two-thirds of breast cancers at diagnosis. These receptors occur at nuclear as well as membrane-associated sites in the cancer cell, and increasing evidence suggests that membrane signaling induced by ER may contribute to the growth regulation of breast cells. Using controlled homogenization and fractionation of MCF-7 human breast cancer cells, the bulk of specific, high-affinity estradiol (E2) binding is found in nuclear fractions. However, a significant portion of specific, high-affinity E2 binding is also enriched in plasma membranes. Electrophoresis of this membrane preparation allowed detection of a primary 67-kD protein and a secondary 46-kD protein recognized by estrogen and by monoclonal antibody directed to the ligand-binding domain of the nuclear form of ER (ER-MAb). To determine if these proteins co-localize to caveola-related structures, a detergent-free method was used to purify caveola-related membrane fractions. Electrophoresis and Western Blot analysis of caveola-related fractions using ER-MAb show a prominent 46-kD protein and a 67-kD protein, as well as proteins of larger molecular size at 100-kD and 130-kD. These ER-Mab-reactive proteins co-purify with flotillins, caveola-related integral membrane proteins, in MCF-7 cells and with both flotillins and HER-2/neu growth factor receptor in MCF-7 cells with overexpression of HER-2/neu oncogene. Co-localization of ER-Mab-reactive proteins and flotillins was also observed at the surface membrane of MCF-7 cells using a confocal microscopy approach. Estrogen-induced growth of MCF-7 cells *in vitro* correlated well with acute hormonal activation of mitogen-activated protein kinase (MAPK) and AKT kinase signaling. In addition, treatment of MCF-7 cells with ER-MAb blocked E2-stimulated growth *in vitro*, and this antiproliferative effect was accompanied by antibody-induced suppression of the rapid, E2-promoted activation of MAPK and AKT kinase. Estrogen-induced growth of human breast cancer xenografts in nude mice was also significantly reduced by treatment *in vivo* with ER-MAb. Thus, activation of membrane-associated forms of ER appear to contribute to the growth of breast cancers. Localization of these ER-like proteins in caveola-related structures associated with the plasma membrane makes them more readily accessible for interaction with specialized membrane signaling proteins and for cross-communication with peptide receptors. The concept of estrogens acting via both nuclear and membrane-associated receptors, likely by a positive feed-forward, synergistic mechanism, presents a new challenge and a unique opportunity for the development of more effective antiestrogen therapies.

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Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells

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Membrane-associated binding sites for estrogen may mediate rapid effects of estradiol-17 β that contribute to proliferation of human breast cancers. After controlled homogenization and fractionation of MCF-7 breast cancer cells, the bulk of specific estradiol binding is found in nuclear fractions. However, a significant portion of specific, high-affinity estradiol-17 β binding-sites are also enriched in plasma membranes. These estradiol binding-sites co-purify with 5'-nucleotidase, a plasma membrane-marker enzyme, and are free from major contamination by cytosol or nuclei. Electrophoresis of membrane fractions allowed detection of a primary 67-kDa protein and a secondary 46-kDa protein recognized by estradiol-17 β and by a monoclonal antibody directed to the ligand-binding domain of the nuclear form of estrogen receptor. Estrogen-induced growth of MCF-7 breast cancer cells *in vitro* was blocked by treatment with the antibody to estrogen receptor and correlated closely with acute hormonal activation of mitogen-activated protein kinase and Akt kinase signaling. Estrogen-promoted growth of human breast cancer xenografts in nude mice was also significantly reduced by treatment *in vivo* with the estrogen receptor antibody. Thus, membrane-associated forms of estrogen receptor may play a role in promoting intracellular signaling for hormone-mediated proliferation and survival of breast cancers and offer a new target for antitumor therapy. *Oncogene* (2001) 20, 5420–5430.

Keywords: estrogen receptor; breast cancer; plasma membrane; MAP kinase; Akt kinase

Introduction

The growth of breast cells is normally regulated by hormones such as estrogen which bind estrogen receptors (ER). These receptors are present in more than two-thirds of breast cancers at diagnosis (Henderson *et al.*, 1988). According to prevailing

theories of hormone action, estradiol-17 β (E₂ β) diffuses passively across plasma membranes and binds with nuclear receptors (Evans, 1988; Murdoch and Gorski, 1991). The newly formed E₂ β -receptor complex may regulate gene expression by binding estrogen-responsive elements in target genes or by modifying transcription via interaction with other nuclear proteins (McKenna and O'Malley, 2000). These nuclear events are believed to culminate in promotion of cell growth. However, in addition to this mechanism, many rapid effects of estrogen with an onset in seconds have been documented in breast (Aronica *et al.*, 1994; Levin, 1999) and other tissues (Szego and Pietras, 1984; Ramirez and Zheng, 1996; Mendelsohn and Karas, 1999; Watson and Gametchu, 1999). The time course of these events suggests that they do not require precedent gene activation. Rather, many rapid effects of estrogens may be due to activation of membrane-associated signaling pathways.

There is increasing evidence that membrane-associated forms of steroid hormone receptors exist and participate in activation of signaling pathways associated with gene regulation (Szego and Pietras, 1984; Ramirez and Zheng, 1996; Levin, 1999; Watson and Gametchu, 1999). Estrogens are known to trigger rapid stimulation of guanylate and adenylate cyclases (Szego and Davis, 1967; Aronica and Katzenellenbogen, 1993; Aronica *et al.*, 1994; Levin, 1999), Ca²⁺ flux (Pietras and Szego, 1975), nitric oxide synthase (Mendelsohn and Karas, 1999) and protein phosphorylation (Migliaccio *et al.*, 1996). In addition, E₂ β activates within seconds mitogen-activated protein kinase (MAPK) signaling cascades in responsive tissues (Migliaccio *et al.*, 1996). Although activation of these pathways is generally considered to be restricted to transmembrane receptors for peptide hormones and growth factors, the presence of high-affinity receptors for E₂ β associated with the surface membrane of target cells was first reported more than two decades ago (Pietras and Szego, 1977). Proteins immunoreactive with antibodies to nuclear ER occur at the surface membrane of cells that exhibit rapid biologic responses to E₂ β (Pappas *et al.*, 1995; Chambliss *et al.*, 2000; Russell *et al.*, 2000). Chinese hamster ovary (CHO) cells transfected with expression constructs for ER have also been shown to express a portion of ER protein on their surface and to

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respond to estrogen with rapid membrane-initiated effects (Razandi *et al.*, 1999). These data have led to a growing recognition that the genomic model of estrogen action is incomplete and must be expanded to include membrane-associated receptors as a component of cell signaling (Pietras and Szego, 1977; Nemere and Farach-Carson, 1998; Levin, 1999; Watson and Gametchu, 1999).

In the present study, we demonstrate that human breast cancer cells contain a membrane-associated binding site for estrogen that closely resembles nuclear ER. Activation of this membrane-associated receptor appears to promote rapid stimulation of MAPK and Akt kinase signaling and later cell proliferation. Biologic activity of the membrane-associated receptor can be diminished *in vitro* and *in vivo* by antibody directed against the ligand-binding domain of nuclear ER. The results suggest that estrogens may initiate membrane-associated signaling events leading to modulation of the growth and survival of breast cancers.

Results

Enrichment of high-affinity binding-sites with specificity for $E_2\beta$ in breast cancer cell plasma membranes

To confirm earlier reports of membrane binding-sites for $E_2\beta$ (Szego and Pietras, 1984; Berthois *et al.*, 1986; Pappas *et al.*, 1995; Ramirez and Zheng, 1996; Levin, 1999; Watson and Gametchu, 1999), we measured specific [3H] $E_2\beta$ binding in subcellular fractions of MCF-7 cells after controlled cell homogenization and fractionation (Pietras and Szego, 1979, 1980). With recovery of more than 97% of total $E_2\beta$ binding found in homogenates of MCF-7 cells, specific [3H] $E_2\beta$ binding was distributed among crude nuclear, microsomal, mitochondria-lysosome and cytosol fractions (Figure 1a). After purification of plasma membranes from the crude nuclear fraction by use of discontinuous-sucrose density gradient centrifugation, the PM fraction showed enhanced activity of 5'-nucleotidase, a plasma membrane marker enzyme, to about 23-times that of homogenate (Figure 1a,b). Specific [3H] $E_2\beta$ binding in plasma membranes was enriched to 28-times homogenate activity and represented 22% of homogenate binding. This data shows that specific $E_2\beta$ binding co-purifies with a plasma membrane marker protein in membrane fractions from breast cancer cells. LDH activity, highly enriched in cytosol, is not significantly detected in PM (Figure 1a,b). In addition, cell DNA recovery was $94 \pm 3\%$ of homogenate levels in nuclear fractions, and no DNA was detected in PM fractions (data not shown). Binding of [3H] $E_2\beta$ by PM fractions from MCF-7 cells was analysed further in equilibrium binding studies (Figure 2). Samples of PM were exposed to [3H] $E_2\beta$ concentrations ranging from 1×10^{-10} to 5×10^{-9} M. As shown in Figure 2a, all samples with [3H] $E_2\beta$ alone retain greater amounts of hormone than paired samples in which [3H] $E_2\beta$ was present together with a 100-fold molar excess of

unlabeled hormone. The difference between the two curves, representing specific binding of $E_2\beta$, is plotted in Figure 2b. It is evident that binding of hormone by PM is saturable, and Scatchard analyses of specific [3H] $E_2\beta$ binding (*cf.* Pietras and Szego, 1980) show that the dissociation constant for the binding process is 3.6×10^{-10} M. Total binding sites in PM at saturation correspond to approximately 6.7 pmol $E_2\beta$ per mg membrane protein. In comparison with the estradiol binding properties of intact MCF-7 cells, plasma membrane estrogen-binding sites retain high affinity for specific estradiol binding and exhibit significant enrichment of ligand-binding capacity (see Figure 3). Further, ligand specificity of [3H] $E_2\beta$ binding to PM was established by effective suppression by a 100-fold molar excess of unlabeled $E_2\beta$ (Figure 2b, inset). In contrast, [3H] $E_2\beta$ binding by PM was essentially uninfluenced by these levels of estradiol-17 α , progesterone or testosterone.

Identification of estrogen receptor forms in subcellular fractions after gel electrophoresis

To characterize putative estrogen receptor forms associated with PM fractions, samples were subjected to Western blot analysis, and blots were probed either with anti-ER antibody Ab2 or with $E_2\beta$ -POD (Luconi *et al.*, 1999). PM purified from MCF-7 cells show significant enrichment of a primary 67-kDa protein that reacts strongly with antibody Ab2 to LBD of nuclear ER- α (Figure 4a). Similarly, breast cell nuclear fractions are enriched with this protein reactive with ER- α antibody (Figure 4a). The 67-kDa band also shows evidence of specific labeling with $E_2\beta$ -POD (Figure 4b). A secondary band at 46 kDa and minor bands at 62 kDa and 97 kDa were detected in PM and other cell fractions by use of Western blot (Figure 4a) and ligand-blotting (Figure 4b). Using an antibody directed to ER- β , no significant reactivity with proteins at the expected size of 58–62 kDa was found in homogenate, nuclear or plasma membrane fractions of the MCF-7 cells (data not shown).

Rapid effects of $E_2\beta$ and $E_2\beta$ -BSA on activation of MAPK and Akt kinase in breast cancer cells

Post-receptor signal transduction events, such as stimulation of MAPK, extracellular signal-regulated kinase ERK-1 (p44) and ERK-2 (p42) (Migliaccio *et al.*, 1996; Levin, 1999), may contribute to proliferative effects of $E_2\beta$ in breast cells. Thus, we assessed estrogen-induced phosphorylation of MAPK in MCF-7 cells *in vitro*. $E_2\beta$, but not 17 α -estradiol ($E_2\alpha$), promotes phosphorylation of MAPK isoforms, with effects evident within 2 min (Figure 5a). To test whether activation of MAPK by $E_2\beta$ may be mediated by binding of estrogen to membrane-associated receptors, MCF-7 cells were treated with $E_2\beta$ linked to BSA, a macromolecular complex considered to be membrane-impermeant (Berthois *et al.*, 1986; Ramirez and Zheng, 1996; Razandi *et al.*, 1999; Russell *et al.*,

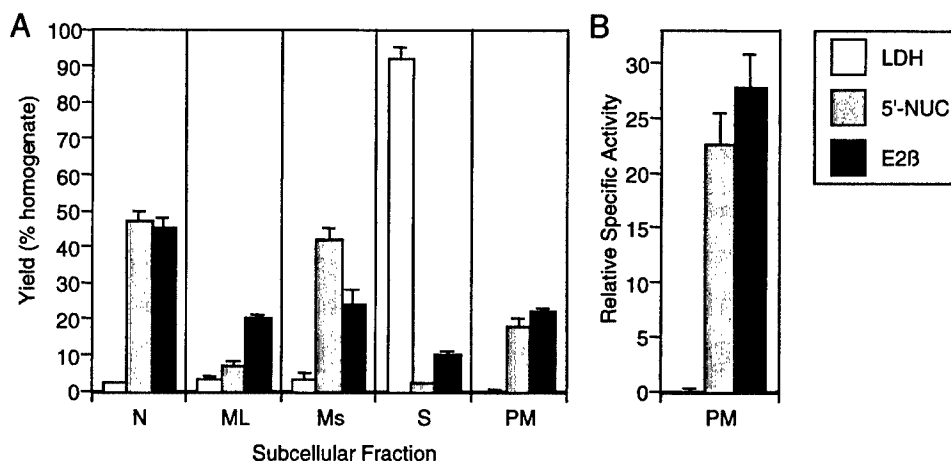


Figure 1 Distribution and relative specific activities of enzymes and specific [3 H]estradiol-17 β binding in plasma membrane and other subfractions of MCF-7 breast cancer cells. Cells were grown in estrogen-free media prior to harvesting, then disrupted using controlled homogenization methods as before (Pietras and Szego, 1979, 1980). (a) The yield of marker enzymes and E $_2$ β binding in each fraction is expressed as a percentage of that in the cell homogenate, with mean \pm s.e.m. of data from three experiments shown. Total recoveries of protein, 5'-nucleotidase (5'-NUC), lactate dehydrogenase (LDH) and specific [3 H]E $_2$ β binding (E $_2$ β) in crude nuclear (N), mitochondria-lysosome (ML), microsome-rich (Ms) and cytosol (S) fractions ranged from 96 to 102% of that in homogenates. Homogenate values averaged 34 ± 2 mg/10 8 cells for protein; 49 ± 2 nmol/min/mg protein for 5'-nucleotidase; 48 ± 4 units/min/mg protein for LDH; and 240 ± 5 fmol/mg protein for specific [3 H]-E $_2$ β binding. (b) Relative specific activity in plasma membrane (PM) represents the specific activity of enzyme or E $_2$ β binding in a given fraction in relation to that in homogenates

2000). Using E $_2$ β -BSA, but not control E $_2$ α -BSA, phosphorylation of MAPK isoforms is again evident within 2 min of steroid administration. Incubation of cells with antibody against LBD of ER (Ab2) inhibited MAP kinase phosphorylation induced by E $_2$ β or E $_2$ β -BSA. Similarly, we assessed signaling via the phosphatidylinositol-3 kinase (PI3K)/Akt pathway after treatment of MCF-7 cells with E $_2$ β or E $_2$ β -BSA. Both ligands induced significant activation of Akt kinase (Figure 5b), and inhibition of estrogen-induced effects occurred when cells were preincubated with ER antibody (Ab2), pure antiestrogen (ICI 182,780) or the PI3K inhibitor, LY 294002.

To assess the potential for MCF-7 cell activation by free estradiol liberated from E $_2$ β -BSA, we transfected MCF-7 cells with an ERE-CAT reporter gene as before (Pietras *et al.*, 1995). Cells were exposed *in vitro* to free estradiol-17 β or to E $_2$ β -BSA for only 10 min, then washed and incubated further. After 24 h, ERE-CAT reporter gene activity was measured. Short-term treatment with free estradiol-17 β stimulated a marked increase in reporter gene activity ($P < 0.001$), but E $_2$ β -BSA elicited no significant effect (Figure 6).

Since interaction of E $_2$ β -BSA with plasma membrane binding-sites may be required for intracellular signaling (Berthois *et al.*, 1986; Ramirez and Zheng, 1996; Razandi *et al.*, 1999; Russell *et al.*, 2000), we evaluated binding of fluorescein-labeled E $_2$ β -BSA (E $_2$ β -BSA-FITC) in MCF-7 cells. E $_2$ β -BSA-FITC binds at the surface of 77% of MCF-7 cells (Figure 7a), while only minimal background fluorescence is found among cells incubated with control ligand, BSA-FITC (Figure 7b). In additional control studies, ER-positive ZR75-1 breast cancer cells, as MCF-7 cells, show retention of E $_2$ β -BSA-FITC at the cell surface, but ER-negative

MDA-MB-231 breast cancer cells or COS-7 cells do not show significant binding of E $_2$ β -BSA-FITC at the external membrane (data not shown). On flow cytometric analysis (Figure 7e), the E $_2$ β -BSA-FITC complex shows evidence of ligand specificity, with significant reduction ($P < 0.01$) of E $_2$ β -BSA-FITC binding by competition with equimolar amounts of free E $_2$ β , E $_2$ β -BSA, tamoxifen or ICI 182, 780, while the related steroid congener, progesterone, is not effective. Surface binding of E $_2$ β -BSA-FITC is also significantly diminished by competition with antibody to the LBD of nuclear ER, suggesting some immunologic identity of the membrane site with nuclear ER (Figure 7c,e). As expected, after permeabilization of cells by disruption of plasma membrane with detergent, intense labeling of ER in cell nuclei is found and occurs in 96% of breast cancer cells (Figure 7d).

Inhibition of cell growth in vitro by antibody to ligand-binding domain of ER- α

Since antibodies to cell surface growth factor receptors are sometimes effective in blocking tumor cell growth (Pietras *et al.*, 1994), the antiproliferative activity of antibodies to ER- α was evaluated using MCF-7 cells *in vitro*. The estrogen-dependent MCF-7 cells show enhanced proliferation after treatment with E $_2$ β , but not E $_2$ α (Figure 8a). However, prior exposure to LBD Ab1 or LBD Ab2 elicits a significant reduction ($P < 0.05$) in the growth response to E $_2$ β (Figure 8a).

Since some recent studies suggest that the proliferative response to E $_2$ β is committed within 1 min and is evoked by activation of only a small fraction ($\leq 5\%$) of ER (Chun *et al.*, 1998), we assessed the growth of breast cells after brief treatment with E $_2$ β -BSA. MCF-7

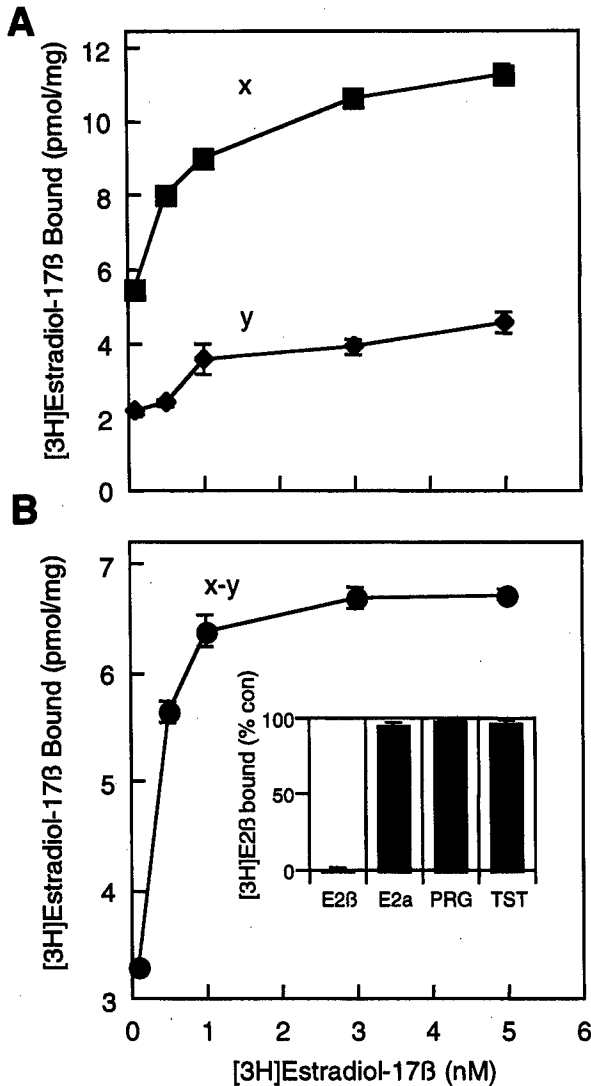


Figure 2 Binding of [³H]estradiol-17β by plasma membranes from MCF-7 human breast cancer cells. (a) Plasma membranes were incubated in Ca²⁺-free medium with 0.25 M sucrose with proteinase inhibitors at 50 μg membrane protein/2.5 ml for 2 h at 4°C with the concentrations of [³H]E₂β given alone (curve x) or in the presence of a 100-fold molar excess of unlabeled E₂β plus [³H]E₂β (curve y). (b) This curve shows the difference between the two curves in (a) and represents the specific binding of hormone by plasma membranes. In the inset, ligand specificity of [³H]estradiol-17β binding was determined by incubation in the presence of a 100-fold molar excess of competing steroidal compounds: E₂β, E₂α, progesterone (PRG) or testosterone (TST) as indicated in the graph. Values are shown as mean per cent control ± s.e.m. (n = 3)

cells were treated with 0.5 μM E₂β-BSA for only 10 min. Then, cells were rinsed and cultivated in estrogen-free media for an additional 72 h. The results show that E₂β-BSA ($P < 0.001$), but not control E₂α-BSA, stimulates cell growth (Figure 8a). Moreover, the proliferative effect of E₂β-BSA is blocked by treatment of cells with ICI 182,780, a pure antiestrogen ($P < 0.001$) (data not shown), or by prior exposure to anti-ER Ab1 ($P < 0.05$) or Ab2 ($P < 0.001$) (Figure 8a).

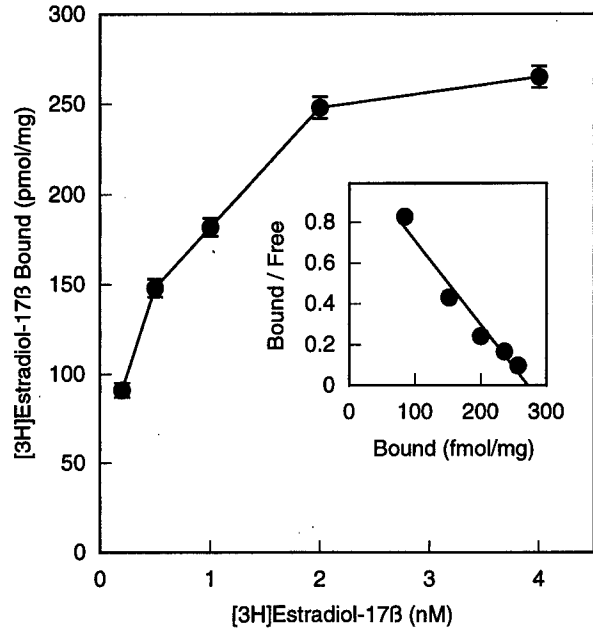


Figure 3 Binding of [³H]estradiol-17β by MCF-7 human breast cancer cells. Specific binding of [³H]estradiol-17β by MCF-7 cells was determined by methods described elsewhere (Pietras and Szego, 1979; Pietras *et al.*, 1995). A 100-fold molar excess of unlabeled estradiol-17β was present with [³H]estradiol-17β in paired samples for determination of displaceable binding. The specific binding of estradiol by plasma membranes is shown. Scatchard analyses of the binding data to determine estrogen-binding capacity (B_{max}) and the affinity of hormone binding (K_d) are shown in the inset. The K_d of estradiol binding to MCF-7 cells was 2.5×10^{-10} M, and the estradiol binding capacity in MCF-7 cells was 270 fmol/mg protein. These values are based on results from three independent experiments

Inhibition of breast tumorigenesis in vivo by antibody to ligand-binding domain of ER-α

The antitumor activity of antibodies to ER-α was evaluated further using MCF-7 tumors *in vivo*. MCF-7 cells were grown as subcutaneous xenografts in female athymic mice primed with E₂β to promote growth of these estrogen-dependent cells (Pietras *et al.*, 1995). Antibody or control treatments were initiated when tumors grew to > 30 mm³. Anti-ER Ab2 was administered in six doses over a 26-day period. The results show that antibody to ER, but not control immunoglobulin, elicits a significant suppression of tumorigenesis of human MCF-7 breast cancer xenografts in female nude mice treated concomitantly with E₂β (Figure 8b).

Discussion

It is generally held that the biologic activity of estrogen in the breast is mediated through receptors that act in breast cell nuclei (Evans, 1988). However, E₂β is also well known to elicit rapid membrane-initiated signaling with an onset in seconds to minutes (Zysek *et al.*, 1981), and these effects are poorly explained by a pure genomic model of hormone action (Szego and Pietras,

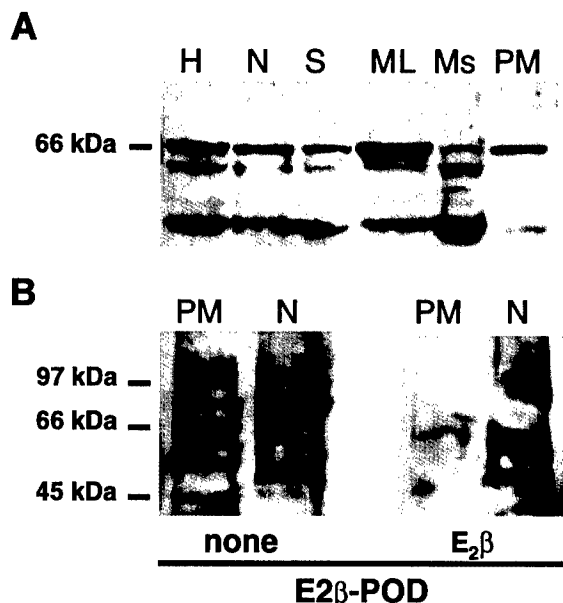


Figure 4 Identification of estrogen receptor in subcellular fractions of MCF-7 cells by Western blot and ligand-blot analyses. Proteins from cell subfractions were analysed by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. (a) Immunoblotting with a monoclonal antibody against the LBD of nuclear ER shows the presence of a major 67-kDa band in the homogenate (H) as well as in nuclear (N), mitochondria-lysosome (ML), microsome (Ms) and cytosol (S) fractions. Notably, a band of similar molecular size also shows enrichment in plasma membrane fractions (PM). (b) Using a ligand-blot approach, binding of E₂β-POD to a 67-kDa band is likewise found to be enriched in plasma membranes (PM) and in nuclear (N) fractions. E₂β-POD binding is shown in the absence (none) and presence (E₂β) of free estradiol-17β at a 10-fold molar excess in order to assess specific steroid binding (Luconi *et al.*, 1999)

1984; Ramirez and Zheng, 1996; Levin, 1999; Watson and Gametchu, 1999). Many studies have provided evidence for a membrane-associated form of ER (Szego and Pietras, 1984; Nemere and Farach-Carson, 1998; Levin, 1999; Watson and Gametchu, 1999). The present work extends these observations in studies with breast cancer cells and suggests that targeting of membrane-associated forms of ER may elicit a potent antitumor effect.

As with affinity-binding strategies used to identify peptide hormone receptors at the cell surface, estrogen-binding sites were identified at the surface of breast cancer cells by exposure to estradiol conjugated with fluorescein-BSA (Nenci *et al.*, 1981; Berthois *et al.*, 1986; Ramirez and Zheng, 1996) or with biotin (Germain *et al.*, 1993). Macromolecule-bound forms of E₂β, such as E₂β-BSA, are generally considered membrane-impermeant and are more water-soluble than free E₂β (Ramirez and Zheng, 1996; Razandi *et al.*, 1999; Russell *et al.*, 2000). These conjugates were first used as immunogens and for affinity purification of nuclear forms of ER (Sica *et al.*, 1973). However, on extended incubation, E₂β-BSA is not stable in solution, especially in the presence of cells, and may release

measurable amounts of free steroid (Steviss *et al.*, 1999). To promote the absence of free E₂β in these experiments, aliquots of E₂β-BSA were preabsorbed with DCC under conditions that remove >99% of free hormone (Russell *et al.*, 2000), and experiments were conducted with only brief exposure of intact cells to E₂β-BSA. In addition, in order to assess the potential for cell activation by free estradiol liberated from E₂β-BSA in our studies, we transfected MCF-7 cells with an ERE-CAT reporter gene and found that E₂β-BSA elicited no significant increase in reporter gene activity. This indicates that, under the conditions of our experiments, neither E₂β-BSA nor dissociated estradiol enters the cell to bind and stimulate the ERE, a finding consistent with prior reports (Watters *et al.*, 1997; Razandi *et al.*, 1999, 2000; Russell *et al.*, 2000). Further, binding of E₂β-BSA to the surface of MCF-7 cells was specific, with little competition by E₂α-BSA or progesterone. About 77% of MCF-7 cells had plasma membrane binding-sites for specific interaction with E₂β-BSA, but the relative concentration of estradiol binding-sites in membrane is substantially less than that found in cell nuclei after cell permeabilization. In additional control studies, cells with no expression of ER-α did not exhibit surface labeling with E₂β-BSA, thus confirming previous reports by Berthois *et al.* (1986).

Despite renewed interest in membrane-associated forms of ER, the identity of these receptors still remains elusive. These receptors may be known membrane molecules with previously unknown binding sites for E₂β, new forms of ER, classical ER complexed with other membrane-associated proteins or truly novel membrane proteins (Szego and Pietras, 1984; Nemere and Farach-Carson, 1998; Levin, 1999; Watson and Gametchu, 1999). Early work on purification of estrogen-binding components from uterus and liver plasma membranes suggested that it was a protein species with high-affinity binding for E₂β and with a molecular size in the range of nuclear ER (Pietras and Szego, 1979, 1980). To determine whether the membrane-associated receptor had antigenic homology with nuclear ER, Pappas *et al.* (1995) used antibodies prepared to different functional epitopes of nuclear ER and demonstrated significant surface labeling in intact pituitary cells by confocal scanning laser-microscopy. Using human endothelial cells, Russell *et al.* (2000) independently confirmed that surface binding-sites for estradiol react specifically with antibodies directed to ER-α. To evaluate the source and distribution of membrane-associated ER, Razandi *et al.* (1999) transfected cDNA for ER-α and ER-β into CHO cells that do not normally express these genes. Expression of a single cDNA encoding either receptor gave rise both to nuclear and membrane ER, suggesting that membrane and nuclear ER derive from a single transcript. The affinity of the receptors for E₂β in both sites was nearly identical, but a greater number of receptors was detected in cell nuclei. In addition, both ER-α and ER-β membrane receptors could

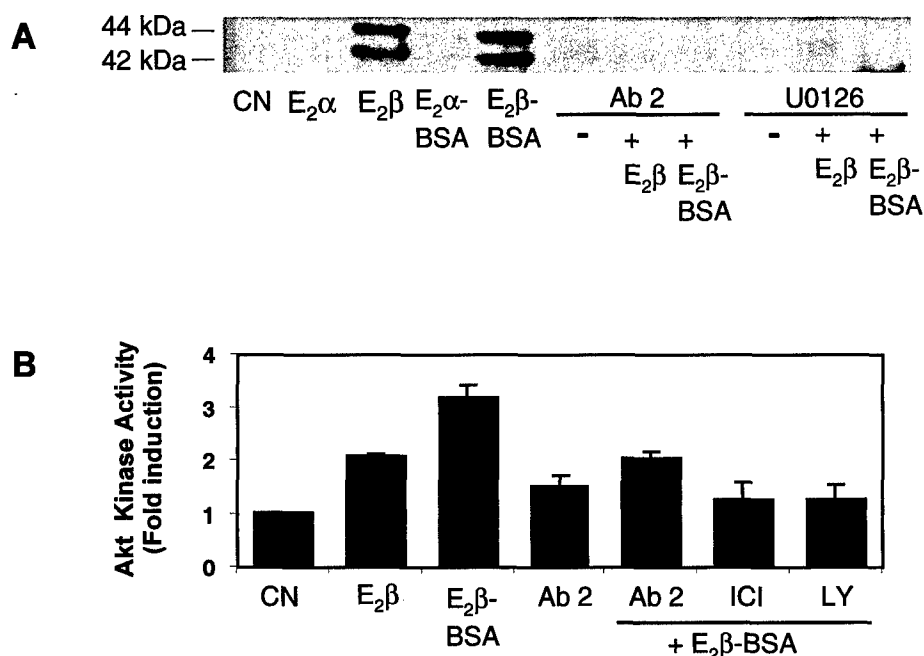


Figure 5 Post-receptor signal transduction induced by estradiol *in vitro*. (a) Treatment of MCF-7 cells with 10 nM estradiol-17 β (E₂β) induces rapid phosphorylation of mitogen-activated protein kinase (MAPK). E₂β, but not 10 nM 17 α -estradiol (E₂α) or vehicle control (CN), promotes phosphorylation of MAPK isoforms, extracellular signal-regulated kinase ERK-1 (p44) and ERK-2 (p42), with effects evident within 2 min. Similarly, MCF-7 cells treated with E₂β covalently linked to BSA (E₂β-BSA, 0.5 μ M), but not to control E₂α-BSA (0.5 μ M), promoted MAPK phosphorylation within 2 min. Prior treatment with antibody to the LBD of ER (Ab2) blocked the expected response to E₂β (Ab2+E₂β) and to E₂β-BSA (Ab2+E₂β-BSA). In addition, cells were preincubated with UO126, a selective inhibitor of MEK1 and MEK2, before treatment with estrogens, and the inhibitor prevented activation of MAPK by E₂β (UO126+E₂β) and by E₂β-BSA (UO126+E₂β-BSA). (b) Akt kinase activation was measured by densitometric analysis of phosphorylated GSK-3 α /β. MCF-7 cells were treated with vehicle (CN) or stimulated with 10 nM estrogen (E₂β) or 0.5 μ M E₂β-BSA for 20 min. Cells were preincubated with anti LBD Ab2 (Ab2), ER antagonist ICI 182, 780 (ICI) or the PI3-kinase inhibitor LY294002 (LY) before addition of E₂β-BSA.

signal to MAPK which was necessary for activating DNA synthesis (Razandi *et al.*, 1999).

In attempting to isolate and purify membrane-associated estrogen-binding proteins from breast cancer cells, this study has begun to further elucidate molecular properties of the membrane-associated receptor in malignant cells. After use of controlled and quantitative cell fractionation procedures to preserve the integrity of subcellular structures (Pietras and Szego, 1979, 1980), the bulk of specific E₂β binding in MCF-7 cells is found in nuclear fractions. However, a significant portion of specific E₂β-binding sites also occur in association with plasma membranes. These E₂β binding-sites co-purify with 5'-nucleotidase, a plasma membrane-marker enzyme, and appear to be free from significant contamination by cytosol or nuclei. The plasma membrane E₂β binding-sites constitute about 20% of total cell binding-sites for the steroid, a level of membrane concentration comparable to that found for other known transmembrane hormone receptors (Bergeron *et al.*, 1986). In addition, monoclonal antibodies against the LBD of nuclear ER can identify membrane-associated ER in MCF-7 cells, a finding consistent with studies with other cell types (Pappas *et al.*, 1995; Razandi *et al.*, 1999; Russell *et al.*, 2000). The primary membrane-associated protein reactive

with antibodies to LBD of nuclear ER- α and with E₂β ligand is 67-kDa, a molecular size comparable to that of nuclear ER- α , but additional protein species, notably at 46-kDa, were also detected (Green *et al.*, 1986; Monje and Boland, 1999). Two forms of ER- α with molecular masses of 67 kDa and about 46 kDa occur in target cells, including vascular endothelial cells (Russell *et al.*, 2000) and MCF-7 cells (Flouriot *et al.*, 2000), and in HeLa cells transfected with ER cDNA (Green *et al.*, 1986). The occurrence of the truncated receptor form may be due, in part, to limited protein degradation or to alternative translation (Flouriot *et al.*, 2000). The smaller receptor form does not appear to be related to ER- β . Antibody directed to ER- β did not react with proteins at the expected size of 58–62 kDa in homogenate, nuclear or plasma membrane fractions of MCF-7 cells (see Fuqua *et al.*, 1999). This is consistent with several reports that find little to no ER- β transcripts in MCF-7 cells (Register and Adams, 1998; An *et al.*, 2001). Finally, a minor band at 97-kDa was detected in plasma membrane and other cell fractions by use of immunoblotting with antibodies to ER- α and with ligand-blotting. This large species may be related to a membrane steroid receptor of high molecular size as reported by others (Chen *et al.*, 1999; Watson and Gametchu, 1999).

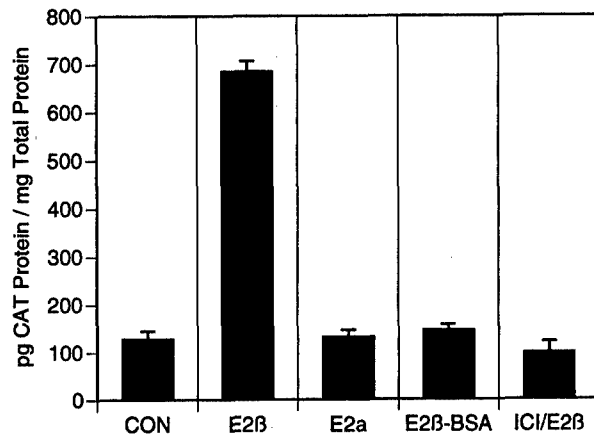


Figure 6 Activation of an ERE-CAT reporter gene by free estradiol-17 β but not by E₂ β -BSA. Using established procedures (Pietras *et al.*, 1995), MCF-7 cells were transfected with a reporter plasmid containing a palindromic estrogen responsive element (ERE) and the chloramphenicol acetyltransferase (CAT) gene, termed ERE-CAT. Transfected cells were treated with free estradiol (1 nM) or with DCC-treated E₂ β -BSA (500 nM) for 10 min, washed extensively and incubated further to 24 h. Thereafter, CAT protein was quantitated in cell extracts and normalized for total protein content in each sample in three independent experiments. In additional control experiments, neither free estradiol nor E₂ β -BSA elicited stimulation of a control ERE reporter gene construct transfected in MCF-7 cells as before (Pietras *et al.*, 1995)

These data suggest that membrane-associated estrogen-binding proteins contain common structural elements, at least in key molecular domains, with ER- α and various splice variants. The classical receptors contain several hydrophobic regions, but it is not known if these are sufficient to allow disposition as an integral membrane protein (Green *et al.*, 1986). However, a distinct E₂ β -binding protein that differs from nuclear ER may also occur (*cf.* Hawkins *et al.*, 2000). Using ER-gene knockout mice, Gu *et al.* (1999) find that some rapid actions of estradiol on kainate-induced currents in neurons still occur and are not inhibited by ICI 182, 780, a pure antagonist of hormone binding to both ER- α and ER- β . However, one difficulty with interpretation of these findings is that uterine tissues from ER-gene knockout mice exhibit up to 10% of estradiol binding present in wild-type uteri (Couse *et al.*, 1995), and the nature of these residual estrogen-binding sites in ER-knockout target cells remains undefined (Flouriot *et al.*, 2000). It is notable that Welshons *et al.* (1993) enucleated MCF-7 cells and found that the resulting cytoplasmic fractions contained about 15% of total cellular ER content, possibly attributable to plasma membrane-associated receptor forms. ER in membranes may also be contained in multimeric complexes with other transmembrane molecules coupled to specific signaling cascades (Mendelsohn and Karas, 1999; Simoncini *et al.*, 2000). This proposal is supported by reports of estrogen receptor forms in 100-nm vesicular invaginations of plasma membrane (Pietras and Szego, 1984) and in association with caveolin and other components

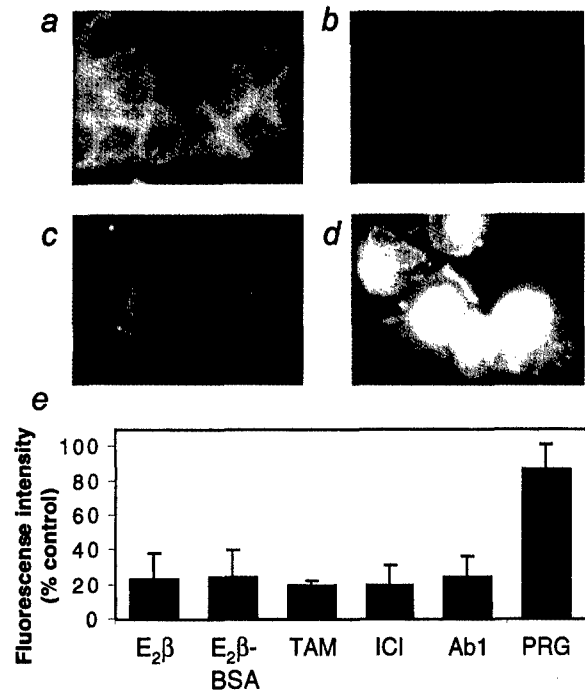


Figure 7 Estradiol-17 β conjugated to fluorescein-labeled albumin (E₂ β -BSA-FITC) shows binding at the surface membrane of MCF-7 breast cancer cells. Cells were labeled with 1 μ M E₂ β -BSA-FITC, a membrane-impermeant complex, to assess membrane binding and then analyzed by fluorescent microscopy and flow cytometry. (a) Active ligand, E₂ β -BSA-FITC, labels surface membranes of MCF-7 cells. (b) Control binding with inactive ligand, BSA-FITC, shows a low level of background cell fluorescence. (c) Surface membrane labeling by E₂ β -BSA-FITC is competitively reduced by co-incubation with antibody to LBD of ER (Ab1). (d) MCF-7 cells were permeabilized with 0.1 % Triton X-100 to allow visualization of ER binding in the nucleus. (e) Flow cytometric analysis of membrane fluorescence with E₂ β -BSA-FITC. Cells were incubated with BSA-FITC for background fluorescence. With 10 000 cells analysed per sample, a significant decrease ($P < 0.01$) in fluorescence intensity was observed when cells were incubated with estrogen (E₂ β), E₂ β -BSA, tamoxifen (TAM), ICI 182, 780 (ICI) or anti ER antibody (Ab1). No significant competition was observed when cells were incubated in the presence of progesterone (PRG). In additional control studies, MDA-MB-231 cells and COS-7 monkey kidney cells with no ER showed no significant binding or retention of E₂ β -BSA-FITC label, while ZR75-1 breast cancer cells with ER expression did show surface binding of the macromolecular complex (data not shown)

of caveolae (Kim *et al.*, 1999; Chambliss *et al.*, 2000), a plasmalemmal microdomain involved in the assembly of signaling complexes. It is clear that further purification of the estradiol-binding component associated with surface membrane will be required to assess the true composition of this molecule.

The estrogen receptor plays a central role in regulating cell proliferation in human breast epithelium (Henderson *et al.*, 1988). Estrogen action leading to growth includes activation of early response genes, cell cycle-regulatory gene products and growth factors (Evans, 1988; Henderson *et al.*, 1988; Murdoch and Gorski, 1991; Weisz and Bresciani, 1993). However, the initial targets of estrogens leading to regulation of

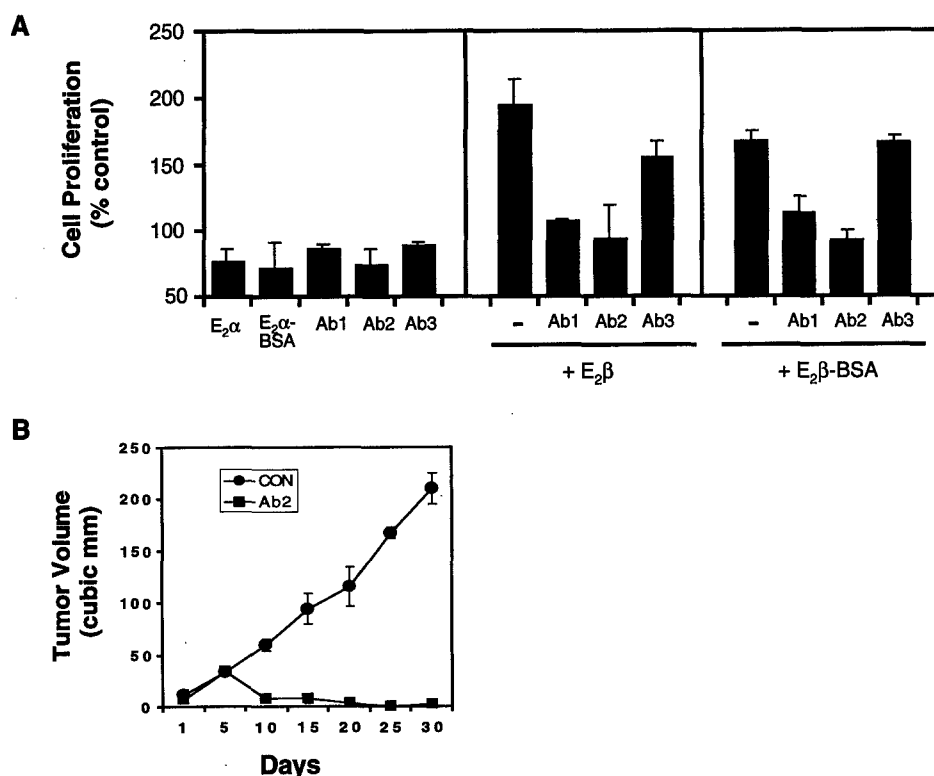


Figure 8 Inhibition of MCF-7 breast cancer cell growth by a monoclonal antibody directed against the LBD of nuclear ER. (a) Cells were incubated *in vitro* for 2 h with anti-ER antibodies directed against the LBD (Ab1 and Ab2) or with a control antibody directed to the D and E-domains of ER (Ab3). Thereafter, E₂β, 17α-estradiol (E₂α), E₂β-BSA or E₂α-BSA were added to cultures for 10 min. Cells were then cultivated further, and final cell numbers were quantitated after 72 h for each treatment group as indicated. Data (mean ± s.e.m.) were collected from at least four independent experiments. (b) Monoclonal antibody directed against the LBD of ER-α reduces growth of human MCF-7 breast tumor cell xenografts *in vivo*. Female nude mice were primed by treatment with E₂β subcutaneously, then inoculated with MCF-7 cells as before (Pietras *et al.*, 1995). After 10–14 days, animals with tumors of comparable size were randomized to treatment groups of 6–8 mice. Treatments included IgG isotype-control antibody (CON) or monoclonal antibody directed against the LBD of ER-α (Ab2) administered intraperitoneally twice weekly for a total of 6 doses. After 26 days, no further antibody treatment was given. Tumor volumes were recorded by micrometer measurements, with results shown as mean ± s.e.m.

the expression of these molecules remain to be identified. As noted above, studies on a variety of target cells suggest that estradiol elicits rapid downstream effects to activate membrane G proteins, inositol phosphate production, adenylate cyclase, calcium, and receptor tyrosine-kinases (Migliaccio *et al.*, 1996; Levin, 1999; Watson and Gametchu, 1999). Estradiol stimulation of breast, bone and vascular endothelial cells is associated with rapid activation of MAPK activity that appears to be independent of transcription (Improta-Brears *et al.*, 1999; Razandi *et al.*, 1999; Russell *et al.*, 2000). These signaling cascades are postulated to promote the later activation of transcription, DNA synthesis and growth (Razandi *et al.*, 1999).

Our studies provide additional confirmation of membrane-initiated signal transduction by ER in breast cancer cells, including interactions with signaling pathways such as MAPK and Akt kinase. Independent reports showing a lack of MAPK activation in the absence of ER (Improta-Brears *et al.*, 1999; Razandi *et al.*, 1999) and the ability of pure antiestrogen, ICI 182,780, to inhibit estrogen-induced MAPK activity in

MCF-7 cells (Improta-Brears *et al.*, 1999) strongly implicate ER in this pathway and define a potentially important link between estradiol and the cell cycle. In the present work, estradiol-17β promotes the phosphorylation of MAP kinase isoforms within 2 min, and these rapid stimulatory effects of free E₂β appear to be equaled by E₂β-BSA *in vitro*. In contrast, steroidal ligands that do not promote cell growth, the biologically-inactive estradiol-17α and E₂α-BSA, do not elicit acute changes in MAP kinase activity. Moreover, the effect of estradiol-17β is abolished by prior incubation of cells with antibody to ER-α or by selective inhibition of the MAP kinase signaling pathway. The ability of a macromolecular antibody to ER to rapidly inhibit estrogen-induced MAP kinase phosphorylation strongly implicates ER in this pathway and suggests an important link between a membrane-associated receptor and cell cycle regulation. In addition, the serine/threonine kinase Akt, a downstream effector of PI3-kinase, has been implicated in cell survival and prevention of apoptosis in MCF-7 cells (Ahmad *et al.*, 1999). Estradiol-17β acutely activates Akt kinase, and the hormone-induced effects

are inhibited by preincubation of cells with ER antibody, ICI 182, 780 or a specific PI3 kinase inhibitor. Again, the ability of antibody to ER and of a pure antiestrogen to inhibit early estrogen-induced Akt kinase activity implicates ER in this pathway and suggest an important association between a membrane-associated receptor and the regulation of a cell survival signaling pathway. Both promotion of cell proliferation and blockade of cell death by membrane-associated estrogen receptors may contribute to the aberrant phenotype of breast cancer cells (see Razandi *et al.*, 2000).

Our studies suggest that activation of membrane-associated forms of ER contribute to promotion of tumorigenesis of breast cancers. Treatment of breast cancers with macromolecular antibodies directed to the LBD of nuclear ER block the growth of tumors that bear functional ER. This finding parallels the report by Norfleet *et al.* (2000) showing that antibodies to ER can modulate rapid prolactin release from pituitary tumor cells with membrane-associated ER. Further study will be required to assess the efficacy of ER antibodies in larger established tumors. Nevertheless, these findings offer support for earlier reports showing that estrogen-induced membrane signaling leads to the later activation of DNA synthesis and cell growth (Razandi *et al.*, 1999). It is likely that primary $E_2\beta$ -induced activation of membrane-associated ER will also affect subsequent hormonal interactions with nuclear ER to promote activation of transcription and cell proliferation. Similarly, the molecular details of cross-communication between estrogen and peptide receptors are beginning to emerge (Szego and Pietras, 1984; Kato *et al.*, 1995; Pietras *et al.*, 1995), and membrane ER may be in a pivotal cellular location to enhance convergence among diverse signaling pathways. Since more than 60% of human breast cancers express ER at diagnosis (Henderson *et al.*, 1988), biologically-based therapies in the form of antiestrogens have been a mainstay in breast cancer treatment. A novel approach to antitumor therapy with blood-borne anti-receptor agents could represent an important addition to available treatment options. In promoting a hypothesis of estrogen action via both nuclear and membrane-associated receptors, this work may lead to development of previously unsuspected antitumor therapies targeted to breast cancers.

Materials and methods

Cell culture and assay of cell proliferation in vitro

MCF-7 human breast cancer cells (ATCC) were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS). For estrogen-free conditions, medium was changed 48 h before experiments to phenol-red free RPMI 1640 with 1% dextran-coated, charcoal-treated (DCC) FBS (Pietras *et al.*, 1995). In experiments using 17β -estradiol 17-hemisuccinate covalently linked to bovine serum albumin ($E_2\beta$ -BSA,

Steraloids, Newport, RI, USA), aliquots of $E_2\beta$ -BSA were preabsorbed with DCC to remove free steroid by established methods (Russell *et al.*, 2000).

To assess effects of ER antibodies on cell proliferation in selected experiments, cells were first incubated with antibodies directed against different domains of ER- α : Ab1, against a segment of the ligand-binding domain (LBD) from amino acids 495–595 (Upstate Biotechnology, Lake Placid, NY, USA); Ab2, against a longer segment of the LBD, from amino acids 302–595 (Neomarkers, Fremont, CA, USA) and Ab3 against segments of the hinge-region, HSP 90- and DNA-binding domains, from amino acids 280–335 (Neomarkers). Incubation with antibodies for 2 h was followed by addition of either 10 nM $E_2\beta$, 100 nM $E_2\alpha$ (17α -estradiol, Steraloids), 0.5 μ M $E_2\alpha$ -BSA (6 keto- 17α -estradiol 6-(o-carboxymethyl)oxime:BSA, Steraloids) or 0.5 μ M $E_2\beta$ -BSA for 10 min. After 72 h, cells were counted to estimate rates of cell proliferation, using data from four independent experiments.

Specific binding of estradiol- 17β in breast cancer cells

Specific $E_2\beta$ binding was assessed in MCF-7 cells using [$2,4,6,7,16,17$ - 3 H]estradiol- 17β (NEN, Boston, MA, USA) as reported previously (Pietras and Szego, 1979, 1980; Pietras *et al.*, 1995). A 100-fold molar excess of unlabeled estradiol- 17β was present with [3 H]estradiol- 17β in paired samples for determination of displaceable binding.

Cell homogenization and subcellular fractionation

Cell fractionation was done as before with methods designed to preserve the integrity of subcellular structures (Pietras and Szego, 1979, 1980; Pietras *et al.*, 1995). In brief, cells were harvested with ice-cold Versene in the presence of protease inhibitors, then homogenized using a Dounce homogenizer. Whole homogenate (H) was filtered through nylon mesh and centrifuged at 1000 g for 10 min to yield crude nuclear (N) and post-nuclear supernate fractions. The N fraction was resuspended in 31% sucrose in buffer, loaded on top of a discontinuous sucrose density-gradient and centrifuged at 67 000 g for 2 h. Plasma membranes occurred predominantly at $\rho=1.13$ – 1.16 (PM) (Pietras and Szego, 1979). The postnuclear supernatant was centrifuged at 15 000 g for 30 min, with the resulting pellet representing the mitochondria-lysosome fraction (ML). The supernate was centrifuged at 105 000 g for 1 h to yield the microsomal pellet fraction (Ms) and the soluble cytosol fraction (S). Extracts from cell membranes were solubilized as before (Monje and Boland, 1999). Protein was quantitated using the BCA-200 Protein Assay Kit (Pierce, Rockford, IL, USA).

Analyses of enzyme activity, DNA, estrogen receptor and [3 H]estradiol- 17β binding assay in subcellular fractions

Activity of 5'-nucleotidase (EC 3.1.3.5) was determined by established methods, with specific activities given as nmol/min/mg (Pietras and Szego, 1979, 1980). Activity of lactate dehydrogenase (LDH) was assessed as before (Weiler and Wiebe, 2000). Relative specific activity represents the specific activity of enzyme in a given fraction in relation to that in homogenate. DNA was determined by established methods (Pietras and Szego, 1980). Specific $E_2\beta$ binding was assessed in cell fractions using [$2,4,6,7,16,17$ - 3 H] estradiol- 17β (NEN) as reported previously (Pietras and Szego, 1979, 1980). To characterize putative estrogen receptor forms associated with PM fractions, membrane proteins were separated by SDS-

PAGE and then transferred to a nitrocellulose membrane for immunodetection by Western blot. The blots were probed as before (Pietras *et al.*, 1995) with ER- α antibodies, Ab1 (Upstate Biotechnology) or Ab2 (Neomarkers), ER- β antibody (PA1-310B; Affinity Bioreagents, Golden, CO, USA) or E $_2$ - β -POD (Luconi *et al.*, 1999).

Determination of p44/42 MAPK and Akt kinase activity

Cells were maintained in estrogen-free conditions 48 h before the experiment. In selected studies, cells were pre-incubated 90 min with U0126 (25 μ M), a selective inhibitor of MEK1 and MEK2 (Favata *et al.*, 1998) or for 2 h with anti-LBD Ab-2 (10 μ g/ml) before treatment with estrogens. Protein samples were separated by SDS-PAGE and then transferred to a nitrocellulose membrane for immunodetection with anti-phospho-p44/p42 MAP kinase (Thr202/Tyr204) polyclonal antibody (New England Biolabs, Beverly, MA, USA), using the Pierce Western blotting system. Akt activity was measured by Western blot using the Akt kinase assay kit (Cell Signaling Technology, Beverly, MA, USA). After growth in estrogen-free conditions, cells were pre-incubated with anti-LBD Ab-2 (10 μ g/ml), anti-PI(3) kinase inhibitor LY294002 (10 μ M) (Haynes *et al.*, 2000) or ICI 182,780 (1 μ M) (Astra Zeneca, Newark, DL, USA), followed by treatment with 10 nM E $_2$ or 0.5 μ M E $_2$ -BSA for 20 min. Lysates were incubated overnight with anti-Akt kinase antibody. Thereafter, immunoprecipitates were processed for assay of Akt kinase activity according to recommendations of the manufacturer. Akt activity was assessed by densitometric analysis of phosphorylated GSK-3 using the public domain NIH Image program.

Transfection of cells with ERE-CAT reporter gene constructs

A reporter plasmid containing a palindromic estrogen responsive element (ERE) and the chloramphenicol acetyltransferase (CAT) gene was used in these studies and is termed ERE-CAT (Pietras *et al.*, 1995). MCF-7 cells were prepared and transfected using established procedures (Pietras *et al.*, 1995). CAT protein was quantitated in cell extracts using a non-radioactive enzyme-linked immunosorbent assay (5 Prime-3 Prime, Boulder, CO, USA), with about 50 pg of CAT protein per ml of cell extract found to be the lower limit of detection. CAT reporter activity was normalized for the protein content in each sample.

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Membrane labeling with estradiol and flow cytometry

Single cell suspensions of MCF-7 cells from estrogen-free cultures were obtained using Versene (GIBCO BRL, Rockville, MD, USA). Cells were incubated at 4°C for 15 min with 1 μ M fluorescein isothiocyanate (FITC)-labeled BSA with covalently-attached 17 β -estradiol-hemisuccinate (E $_2$ -BSA-FITC) (Berthois *et al.*, 1986). In selected control experiments, ER-positive ZR75-1 human breast cancer cells (ATCC) or ER-negative MDA-MB-231 human breast cancer cells (ATCC; Berthois *et al.*, 1986) or COS-7 monkey kidney cells (ATCC) were labeled under similar conditions. For competition studies, MCF-7 cells were incubated 5 min with 100 nM E $_2$, 1 μ M E $_2$ -BSA, 1 μ M ICI 182,780, 100 nM progesterone or 10 μ g/ml ER antibody Ab1. A sample was analysed by microscopy, and the remainder was used for flow cytometry using a FACScan with Cell Quest software (Beckton Dickinson, Franklin Lakes, NJ, USA). To facilitate nuclear staining, some cells were permeabilized with 0.1% Triton X-100 (Razandi *et al.*, 1999).

Human tumor xenografts in nude mice

MCF-7 cells were inoculated subcutaneously at 5×10^7 cells/animal in the mid-back region of 3-month-old female athymic mice (Charles River, Wilmington, MA, USA) primed with E $_2$ in a biodegradable binder as before (Pietras *et al.*, 1995). Treatment was initiated when tumors grew to >30 mm³. Animals were randomized by weight and tumor size at the start of the experiment, with 6–8 animals included in each treatment group. Antibody and control solutions were administered by intraperitoneal injection. Anti-ER LBD Ab2 was given at a dose of 3.5 mg/kg in 6 doses at 4-day intervals (over 26 days). Control injections with mouse IgG₁ (Pharmingen, San Diego, CA, USA) were given on an identical treatment protocol.

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Steroid Hormone Receptors in Target Cell Membranes

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Numerous reports of rapid steroid hormone effects in diverse cell types cannot be explained by the generally prevailing theory that centers on the activity of hormone receptors located exclusively in the nucleus. Cell membrane forms of steroid hormone receptors coupled to intracellular signaling pathways may also play an important role in hormone action. Membrane-initiated signals appear to be the primary response of the target cell to steroid hormones and may be prerequisite to subsequent genomic activation. Recent dramatic advances in this area have intensified efforts to delineate the nature and biologic roles of all receptor molecules that function in steroid hormone-signaling pathways. This work has profound implications for our understanding of the physiology and pathophysiology of hormone actions in responsive cells and may lead to development of novel approaches for the treatment of many cell proliferative, metabolic, inflammatory, reproductive, cardiovascular, and neurologic defects.

Key Words: Steroid hormone action; plasma membrane; receptor.

Introduction

The broad physiologic effects of steroid hormones in the regulation of growth, development, and homeostasis have been known for decades. Often, these hormone actions culminate in altered gene expression (1), which is preceded by nutrient uptake and other preparatory changes in the synthetic machinery of the cell (2). Owing to certain homologies of molecular structure, specific receptors for steroid hormones, vitamin D, retinoids, and thyroid hormone are often considered a receptor superfamily. The actions of ligands in this steroid receptor superfamily are commonly postulated to be mediated by receptors in the cell nucleus. On binding ligand, nuclear receptors associate with target

genes and permit selective transcription. This genomic mechanism is generally slow, often requiring hours or days before the consequences of hormone exposure are evident. However, steroids also elicit rapid cell responses, often within seconds. The time course of these acute events parallels that evoked by peptide agonists, lending support to the conclusion that they do not require precedent gene activation (2-5). Rather, many rapid effects of steroids, which have been termed *nongenomic*, appear to be owing to specific recognition of hormone at the cell membrane. Although the molecular identity of binding sites remains elusive and the signal transduction pathways require fuller delineation, there is mounting evidence that steroid action is initiated by plasma membrane receptors.

A current challenge is to determine the relation of rapid responses to steroid hormones to intermediate and long-term effects. Some questions that arise in this context include the following: Is specific membrane binding responsible merely for cellular entry of the hormone? Do plasmalemmal receptors escort ligand to the nucleus? Are the membrane binding sites coupled to rapid signal transduction systems that also act in concert with nuclear transcription factors? Are the membrane receptors identical to nuclear receptors, modified forms, or entirely different entities? This review explores these important issues. In preparing this work, more than 1200 references providing significant evidence for rapid steroid actions and for membrane forms of steroid receptors were identified. Only a fraction of these citations can be presented here, and the reader is referred to several recent reviews in this area (3-7).

Estrogens

As with other steroid hormones, biologic activities of estrogen in breast, uterus, and other tissues are considered to be fully mediated by a specific high-affinity receptor in cell nuclei. Estrogens are accumulated and retained in responsive cells, and it has been commonly assumed that the steroid diffuses passively to intracellular receptors. However, estradiol is a lipophilic molecule that partitions deep within the hydrocarbon core of lipid bilayer membranes, even those devoid of relevant receptors (3). Several investigations now demonstrate that steroid hormones enter target cells by a membrane-mediated process that is saturable

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Table 1
Brief Chronology of Selected Reports Documenting Occurrence and Activity of Membrane Steroid Hormone Receptors^a

Steroid	Year	Observation	Reference
Estradiol	1967	Elevation of uterine cAMP by estrogen within seconds	11
	1975	Rapid endometrial cell calcium mobilization by estrogen	9
Corticosterone		Binding to plasma membranes of rat liver	108
Estradiol	1976	Effects on electrical activity of neurons	20
	1977	Specific plasma membrane binding sites for estrogen	16
Cortisol		Electrophysiologic effects on neurons	21
Progesterone	1978	Induction of oocyte maturation by steroid linked to a polymer	29
Estradiol	1979	Increased proliferation of cells with membrane ER	17
	1980	Molecular properties of ERs in liver plasma membrane	13
Vitamin D	1981	Rapid intestinal cell calcium uptake	109
Progestin	1982	Specific binding to oocyte surface and role in meiotic maturation	30
		Steroid receptor of 110 kDa on oocyte surface by photoaffinity labeling	31
Corticosterone	1983	Binding to synaptic plasma membranes	50
Estradiol	1983	Increase in density of microvilli at endometrial cell surface within seconds	112
	1984	Primary internalization of ER in endometrial plasma membrane vesicles	104
Thyroid hormone	1985	Characterization of plasma membrane binding sites	47
Estradiol	1986	High-affinity binding sites in breast cancer cell plasma membranes	26
		Altered breast cell membrane potential, density of microvilli within seconds	110
Glucocorticoid	1987	Correlation between membrane receptor and apoptosis in lymphoma cells	53
Vitamin D	1989	Rapid activation of phospholipase C (PLC) in rat intestine	5,14
		Activation of calcium channels in osteoblasts	63
Thyroid hormone		Rapid induction of glucose uptake	42
Progesterone	1990	Stimulation of calcium influx in human sperm	33
	1991	Calcium uptake mediated by sperm cell surface-binding sites	
		Action at plasma membrane of human sperm	34
Corticosterone		Correlation of neuron membrane receptors with behavior in newts	51
Aldosterone		Rapid effects on Na ⁺ /H ⁺ exchange	111
Glucocorticoid	1993	Antigenic similarity between membrane and intracellular receptors	54
Estradiol		Binding and stimulation of HER-2 membrane receptor	90
	1994	Activation of adenylate cyclase signaling pathways	12
Vitamin D		Isolation of a plasma membrane receptor from chick intestine	88
Aldosterone		Identification of membrane receptor in human lymphocytes	86
Estradiol	1995	Membrane receptor with antigenic identity to nuclear receptor	7,78
		Greater nongenomic responses of membrane receptor-enriched neural cells	
Androgen		Rapid increase in cytosolic Ca ⁺⁺ in Sertoli cells	36
Estradiol	1997	Membrane action and PLC regulation	14
		Isolation of membrane binding-proteins from rat brain	81
Vitamin D	1998	Blocking of hormone activation of PKC by antibody to membrane receptor	65
Estradiol	1999	Rapid Ca ⁺⁺ mobilization required for activation of MAPK	10
		Rapid actions in neurons from ER α knockout mice	94
		Reduction of membrane ER expression by antisense to nuclear ER	80
		Membrane and nuclear ER α , and ER β , each expressed from single transcript	25
		Activation of G-proteins, IP ₃ , adenylate cyclase, and MAPK by membrane ER	
Androgen		Rapid activation of MAPK pathway in prostate	37
Progesterone		Cloning and expression of binding protein from liver microsomal membrane	85
Vitamin D	2000	Ligand-induced nuclear translocation of plasma membrane receptor	89
Estradiol		Surface receptor in endothelial cells recognized by monoclonal ER α antibody	79
		Interaction of ER α with regulatory subunit of phosphatidylinositol-3-OH kinase	113
		Rapid tyrosine phosphorylation of Raf-1 and activation of MAPK	114
		resulting in prolactin gene expression in pituitary cells	

^aMore than 1200 publications on membrane steroid receptors have appeared in the past 30 yr. Of these, only representative examples are listed here. The potential roles of alternate (25) or variant (56) forms of steroid hormone receptors and other membrane-signaling molecules (90,94) remain to be clarified.

and temperature dependent (3,8). Moreover, it is well established that estrogen can trigger in target cells rapid surges in levels of intracellular messengers, including calcium (9, 10) and cyclic adenosine monophosphate (cAMP) (11,12), as well as activation of mitogen-activated protein kinase

(MAPK) (13) and phospholipase (14) (Table 1). These data have led to a growing consensus that the traditional genomic model of estrogen action does not explain the rapid effects of estrogens and must be expanded to include membrane receptors as a component of cell signaling (2-7,15).

The first unequivocal evidence for specific membrane-binding sites for estradiol-17 β (E_2) was reported in 1977 (16). Intact uterine endometrial cells equipped with estrogen receptor (ER), but not ER-deficient control cells, bound to an inert support with covalently linked E_2 . In addition, target cells that bound could be eluted selectively with free hormone, and cells so selected exhibited a greater proliferative response to estrogens than cells that did not bind (17,18). Further investigations have continued to provide compelling evidence for the occurrence of a plasma membrane form of ER and support for its role in mediating hormone actions (3) (Table 1).

Selye (19) first demonstrated that steroids at pharmacologic concentrations elicit acute sedative and anesthetic actions in the brain. However, electrical responses to *physiologic* levels of E_2 with rapid onset have since been reported in nerve cells from different brain regions (4,20,21). Similarly, certain vasoprotective effects of estrogen appear attributable to membrane receptors (15,22). Estrogen-induced release of uterine histamine *in situ* has long been associated with rapid enhancement of the microcirculation by a process that excludes gene activation (2). Reinforcing these observations are new data detailing the role of nitric oxide (NO) in vascular regulation by estrogen. Normal endothelium secretes nitric oxide, which relaxes vascular smooth muscle and inhibits platelet aggregation. Estrogens elicit abrupt liberation of NO by acute activation of endothelial NO synthase without altering gene expression, a response that is fully inhibited by concomitant treatment with specific ER antagonists (23). This estrogenic effect may be mediated by a receptor localized in caveolae of endothelial cell membranes (24). Such observations require extension, because several independent cell-signaling complexes that appear to participate in signal transduction to the nucleus also associate with caveolar structures (2,3,22).

Estrogen deficiency is associated with significant bone loss, and research on the potential role of membrane ERs in regulating bone mass has increased. Evidence for membrane-binding sites and acute effects of estrogen with an onset within 5 s has been reported in both osteoblasts and osteoclasts (5,13). The effects of estrogens on bone homeostasis also appear to involve rapid activation of MAPK (13), as has also been demonstrated in certain other target cells (10,15,25).

When exposed to E_2 conjugated to fluorescein-labeled bovine serum albumin (BSA), human breast cancer cells exhibit specific surface staining (7,26). Since E_2 -BSA is considered membrane impermeant, these conjugates, developed primarily for use as immunogens and for affinity purification of nuclear ERs, have also been used to assess the membrane effects of estrogen. However, in light of the fact that E_2 -BSA is unstable in solution, especially in the presence of cells and their enzymic products, and releases measurable amounts of free steroid (27), data relying only on the use of estradiol conjugates to test for membrane effects

of steroids need especially careful scrutiny. It is clear that more stable, cell-impermeant derivatives of estradiol should be developed for evaluating membrane receptors.

Progestogens and Androgens

As documented for estrogens, several physiologic effects of progestogens and androgens appear to be regulated, in part, by membrane receptors. Progesterone controls components of reproductive function and behavior. Some of these activities are mediated by interaction with neurons in specific brain regions, and membrane effects appear to be important in this process (4,28). Meiosis in amphibian oocytes is initiated by gonadotropins, which stimulate follicle cells to secrete progesterone. The progesterone-induced G₂/M transition in oocytes was among the first convincing examples of a steroid effect at plasma membrane, since it could be shown that exogenous, but not intracellularly injected, progesterone elicited meiosis and that many progesterone-stimulated changes occurred even in enucleated oocytes (29–32). Moreover, this process may be related to progesterone-induced increments in intracellular Ca⁺⁺ and release of diacylglycerol species that elicit a cascade of further lipid messengers (32).

Progesterone elicits rapid effects on membrane receptors, second messengers, and the acrosome reaction in human sperm (33–35). Assay of acute sperm responses to progesterone in subfertile patients is highly predictive of fertilizing capacity (35). Effects of the steroid, present in the cumulus matrix surrounding the oocyte, appear to be mediated by elevated intracellular Ca⁺⁺, tyrosine phosphorylation, chloride efflux, and stimulation of phospholipases, effects attributed to activation of a membrane-initiated pathway. Indeed, two different receptors for progesterone, apparently distinct from genomic ones, have been identified at the surface of human spermatozoa (35); nevertheless, a monoclonal antibody (MAb) against the steroid-binding domain of human *intracellular* progesterone receptor (PR) inhibits progesterone-induced calcium influx and the acrosome reaction in sperm (35).

As with estrogens and progestogens, androgens promote a rapid increase in cytosolic Ca⁺⁺ in their cellular targets (36). Other effects of androgens that are not attributable to genomic activation include acute stimulation of MAPK in prostate cancer cells (37). The androgen, 5 β -dihydrotestosterone, induces vasodilation of aorta, which may be owing to direct action of the steroid on membranes of smooth muscle cells leading to modulation of calcium channels (38). In osteoblasts, membrane receptors for androgen appear to be coupled to phospholipase C (PLC) via a pertussis toxin-sensitive G-protein that, after binding testosterone, mediates rapid increments in intracellular calcium and inositol triphosphate (IP₃) (39). Of note, Benten et al. (40) report that testosterone elicits Ca⁺⁺ mobilization in macrophages that lack intracellular androgen receptor (AR). These cells

express an apparent G-protein-coupled AR at the cell surface that undergoes agonist-induced internalization.

Thyroid Hormones

Thyroid hormones are well known to regulate energy expenditure and development, and membrane-initiated effects may contribute to these responses. Triiodothyronine (T_3) rapidly stimulates oxygen consumption and gluconeogenesis in liver (41). T_3 also promotes an abrupt increase in uptake of the glucose analog, 2-deoxyglucose, in responsive tissues by augmenting activity of the plasma membrane transport system for glucose (42). In rat heart, T_3 elicits a positive inotropic effect, increasing left ventricular peak systolic pressure, as early as 15 s after hormone (43). In each tissue investigated, alterations in intracellular Ca^{++} induced by thyroid hormone appear to modulate signal transduction to the cell interior (41–44).

Membrane-initiated effects of T_3 have been documented in bone cells by means of inositol phosphate signaling (45), and in brain through calcium channel activation (46). T_3 can also influence other cell processes, including the exocytosis of hormones and neurotransmitters (46), rapid effects that may be attributable to mediation by membrane receptors (44). Although uptake of T_3 can occur concomitantly with receptor-mediated endocytosis of low-density lipoprotein, and likely accompanied by carrier proteins, uptake of T_3 itself has also been reported to occur in numerous tissues by means of a high-affinity, stereospecific, and saturable process (45,47,48), as found for steroid hormones (3,8).

Glucocorticoids

In addition to their long-established effects on mobilization of energy sources by promoting catabolism and the induction of enzymes involved in gluconeogenesis, glucocorticoids have profound effects on neuron signaling and on induction of apoptosis in lymphocytes, phenomena that appear to be membrane-initiated events. Kelly et al. (21) found that glucocorticoids rapidly altered neuron-firing patterns, and many studies have verified these effects (4,6,28). These molecular events lead to glucocorticoid modulation of specific brain functions, such as the rapid response of hypothalamic somatostatin neurons to stress (49). Such abrupt changes in neuron polarization are reinforced by findings of specific, saturable binding of corticosterone to neuron membranes (50,51). Specific, high-affinity corticosterone binding to calf adrenal cortex plasma membrane is also identified by use of the biologically active radioligand [3H]corticosterone (52).

Glucocorticoids also play an important role in the regulation of immune function and inflammation. In lymphoproliferative diseases, glucocorticoids are in wide use as therapeutic agents, but the cellular mechanism leading to the therapeutic effect remains unclear. In several studies using both cell lines and freshly prepared leukemia or lym-

phoma cells, the presence of a membrane receptor for glucocorticoids has been implicated in modulating apoptosis and cell lysis (7,53–55). Moreover, in lymphocytes, the membrane-binding site is antigenically related to the intracellular glucocorticoid receptor (iGR) and may be a natural splice variant form of the intracellular receptor (7,55,56). A potential parallel to the ER transfected in Chinese hamster ovary (CHO) cells (25) is evident.

Aldosterone and Digitalis-Like Steroids

Beyond its classic functions of promoting renal reabsorption of sodium and excretion of excess potassium, aldosterone enhances sodium absorption from colon and urinary bladder. In each tissue, the mineralocorticoid effect is owing to enhanced activity of amiloride-sensitive sodium channels. Aldosterone rapidly augments Na^+/H^+ exchange (6,57). This function is Ca^{++} - and protein kinase C (PKC)-dependent but independent of nuclear receptor activation, transcription, and protein synthesis (6,58). Similarly, “nongenomic” action of aldosterone has also been reported to underlie its acute effects on cardiac function and on sodium transport in vascular smooth muscle cells (6,58).

Digitalis-like compounds are often forgotten members of the steroid superfamily. These plant-derived agents elicit inotropic and chronotropic effects on the heart but also affect many other tissues. Endogenous steroidal ligands, termed *digitalis-like* or *ouabain-like* factors, have been found in sera of humans and other animals with blood volume expansion and hypertension (59,60) and may be released from adrenal cortex (60). These ligands elicit inhibition of membrane-associated Na^+,K^+ -ATPase, likely the principal receptor for these agonists. It is notable that the steroid-binding domain of Na^+,K^+ -ATPase and that of nuclear hormone receptors share significant amino acid sequence homology (61). In addition to membrane actions of these compounds on Na^+,K^+ -ATPase, ouabain-induced hypertrophy in myocytes is accompanied by promotion of Ca^{++} flux and initiation of protein kinase-dependent pathways leading, in turn, to specific changes in transcription and altered expression of early response- and late-response genes (62). Thus, the biologic effects of digitalis-like compounds, long considered the exception to the concept of exclusive genomic influence, may render them more closely integrated with the steroid hormone superfamily than was previously recognized.

Vitamin D Metabolites

Membrane-initiated effects of the seco-steroid hormone, 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$), are well documented in bone and cartilage. In osteoblasts, Caffrey and Farach-Carson (63) elucidated possible connections between rapid effects of $1,25(OH)_2D_3$, requiring milliseconds to minutes, and longer-term effects owing to gene expression. Their laboratory was the first to show activa-

tion of calcium channels by $1,25(\text{OH})_2\text{D}_3$ (63). Calcium, which can signal gene expression through multiple pathways, promotes key phosphorylation events in certain bone proteins (5). Osteoblasts exhibit rapid changes in IP_3 and diacylglycerol in response to vitamin D metabolites via activation of PLC (5,14). Other bone cells with rapid responses to vitamin D metabolites include osteosarcoma cells and chondrocytes (5,64). The latter system is particularly intriguing because chondrocytes elaborate matrix vesicles that appear critical in bone mineralization. The matrix vesicles, which lack nuclei, exhibit specific, saturable binding of $1,25(\text{OH})_2\text{D}_3$, especially when derived from growth zone chondrocytes (65).

Other rapid effects of vitamin D occur in a variety of cell types. Muscle cells respond within seconds to $1,25(\text{OH})_2\text{D}_3$ via several mediators that alter cardiac output in some instances, while acute activation of calcium channels in skeletal muscle promotes contraction (5,66). Of note, in lymphoproliferative disease, $1,25(\text{OH})_2\text{D}_3$ appears to prime monocytic leukemia cells for differentiation through acute activation or redistribution of PKC, Ca^{++} , and MAPK (5,67). In pancreas and intestine, activation of membrane-associated signaling pathways results in vesicular exocytosis. Pancreatic β -cells respond to $1,25(\text{OH})_2\text{D}_3$ with enhanced intracellular Ca^{++} coupled to increased insulin release (68). In intestine, $1,25(\text{OH})_2\text{D}_3$ stimulates exocytosis of transported vesicular calcium and phosphate. These cellular events may be related to vitamin D-promoted alterations in the levels of α -tubulin (5), thereby influencing assembly of microtubules and possibly providing a means for vectorial transport of absorbed ions. Several signal transduction pathways have been found to respond rapidly to exogenous $1,25(\text{OH})_2\text{D}_3$, including activation of protein kinases and promotion of abrupt increments in Ca^{++} , but integration of these signaling cascades with the physiologic response of enhanced ion absorption remains to be established (5,68,69).

Investigations with vitamin D congeners have recently indicated the potential hormonal nature of $24,25(\text{OH})_2\text{D}_3$, once thought to represent merely the inactivation product of precursor $25(\text{OH})\text{D}_3$. Acute effects of $24,25(\text{OH})_2\text{D}_3$ have been observed in bone cells and in intestine; $24,25(\text{OH})_2\text{D}_3$ also inhibits rapid actions of $1,25(\text{OH})_2\text{D}_3$ (5). This may explain why abrupt effects of $1,25(\text{OH})_2\text{D}_3$ often fail to be observed in vivo (70): normal, vitamin D-replete subjects have endogenous levels of $24,25(\text{OH})_2\text{D}_3$ sufficient to inhibit acute stimulation of calcium transport by $1,25(\text{OH})_2\text{D}_3$, thus providing a feedback regulation system (69).

Retinoids

Retinoic acid exerts diverse effects in the control of cell growth during embryonic development and in oncogenesis. It is widely considered that effects of retinoids are mediated through nuclear receptors, including those for retinoic acid as well as retinoid X receptors (1). However,

other retinoid response pathways appear to exist, independent of nuclear receptors (71). Cellular uptake of retinol (vitamin A) may involve interaction of serum retinol-binding protein with specific surface membrane receptors followed by ligand transfer to cytoplasmic retinol-binding protein (72). In this regard, targeted disruption of the gene for the major endocytotic receptor of renal proximal tubules, megalin, appears to block transepithelial transport of retinol (73). It is noteworthy that megalin may also be implicated in receptor-mediated endocytosis of $25(\text{OH})\text{D}_3$ in complex with its plasma carrier (74). In addition, retinoic acid binds mannose-6-phosphate (M6P)/insulin-like growth factor-2 (IGF-2) receptor with moderate affinity and appears to enhance its receptor activity (75). M6P/IGF-2 receptor is a membrane glycoprotein that functions in binding and trafficking of lysosomal enzymes, in activation of transforming growth factor- β , and in degradation of IGF-2, leading to suppression of cell proliferation. The concept of multiple ligands binding to and regulating the function of a single receptor is relatively novel but has important implications for modulating and integrating the activity of seemingly independent biologic pathways.

Properties of Membrane Receptors for the Steroid Superfamily

Despite renewed interest in membrane steroid receptors, the physical identity of receptors with high binding affinity for ligand remains elusive. Isolation and structural characterization of these molecules remains to be accomplished. They may be known membrane components (e.g., enzymes, ion channel subunits, receptors for nonsteroid ligands), with previously unrecognized binding sites for steroids, new forms of steroid hormone receptors, "classic" receptors complexed with other membrane-associated proteins, or truly novel membrane proteins.

Estrogens and Progestogens

Efforts to isolate and purify membrane receptors that mediate rapid effects of steroids are under way in several laboratories (Table 2). Early work on purification of ER from uterus and liver plasma membranes suggested that it was a protein species with high-affinity, saturable binding specific for estradiol- 17β (16,18). The molecular size of solubilized receptor was in the range of intracellular ER (18,76). Other work to isolate plasma membrane estrogen-binding proteins identified the 67-kDa species characteristic of nuclear receptor, but additional proteins of variant size ranging from 28 to 200 kDa were also revealed (77). To determine whether membrane ER had antigenic homology with nuclear ER, Pappas et al. (78) used antibodies prepared to different functional epitopes of intracellular receptor and demonstrated surface labeling in nonpermeabilized rat pituitary cells by confocal scanning laser microscopy. Recent work by Russell et al. (79) has demonstrated, by means

Table 2
Representative Examples of Physical Properties of Membrane-Associated Receptors for Ligands of Steroid Hormone Superfamily^a

Ligand	MW (kDa)	K_d (M)	Binding capacity (fmol/mg protein)	Homology with nR	Tissue	Reference
Estradiol	51–78 ^b	2.8×10^{-10}	526	ND	Rat hepatocytes	18
	105–148 ^c					
	11–67	3.6×10^{-10}	370	ND	Rabbit uterus	77
Progesterin	67			Yes	CHO cell (ER transfected)	25
	110	5×10^{-7}		ND	Amphibian oocyte	30
	110	1×10^{-6}		ND		31
Vitamin D	28,56	6.9×10^{-8}	Variable	ND	Porcine liver	84
	65	7×10^{-10}	240	No	Chick intestine	88
		1.7×10^{-11}	124	No	Rat growth chondrocytes	65
Aldosterone		2.8×10^{-11}	100	No	Rat resting chondrocytes	
	36	1×10^{-8}		ND	Rat osteoblast-like cells	87
	50	1.1×10^{-8}	350	No	Pig liver	86
Glucocorticoids		1×10^{-7}		ND	Rat synapses	50
	97–150	2.4×10^{-7}	384	Yes	S-49 lymphoma cells	55
		5.1×10^{-10}		ND	Amphibian synapses	51
Thyroid hormone	145	2×10^{-9}	320	No	Human placenta	47
		6×10^{-10}		ND	Rat myoblasts	48

^aOnly representative examples of steroid-binding membrane macromolecules are presented here. Please refer to text for additional references. Homology of membrane macromolecules to nuclear receptor forms (nR) is noted; MW, apparent molecular weight; ND, not determined.

^bHigh salt (0.4 M KCl).

^cLow salt (0.01 M KCl).

of monoclonal anti-ER α , that human endothelial cells possess surface-binding sites for estrogen (see Table 1). In evaluating the source and distribution of membrane ER, target cells with expression of ER α were treated with antisense oligonucleotide to nuclear ER α to suppress expression of receptor protein (80). This approach significantly reduced expression of membrane as well as nuclear forms of ER. Using an alternate method to assess receptor origin, Razandi et al. (25) transfected cDNA for ER α and ER β into CHO cells, which do not normally express ER. The transfections resulted in ER expression in both nuclear and membrane fractions, suggesting that membrane and nuclear ER are derived from a single transcript. In addition, both ER α and ER β were expressed in membranes, and both receptors were capable of activating G-proteins, MAPK, as well as DNA synthesis (25). In related studies, the acute stimulation of endothelial nitric oxide synthase (eNOS) by estrogen was reconstituted in COS-7 monkey kidney cells cotransfected with ER α and eNOS, but not by transfection with eNOS alone (23).

Binding molecules for estrogen and progesterone, comprising several molecular species, were isolated from brain synaptosomes by affinity chromatography and characterized by electrophoresis and Western blot (15,81). Microsequencing of one E₂-binding protein indicated that the high-affinity site corresponds to the subunit of an ATPase/ATP synthase. In addition, some studies suggest that estrogen

bound to sex hormone-binding globulin, a plasma protein, also binds with specificity to membrane sites recognizing the liganded transport protein (82). These transport-protein interactions promote cAMP generation via the intermediacy of G-proteins. However, further characterization of receptors for such steroid:protein complexes is not available, and it must be recalled that estrogen is in noncovalent association with its plasma protein carrier and dissociates readily therefrom (83).

Binding of progesterone to plasma membrane of amphibian oocytes is specific, saturable, and temperature dependent (31,32). Photoaffinity labeling with the synthetic progesterin [³H]-R5020, followed by gel electrophoresis, revealed progesterin binding to both 80- and 110-kDa proteins in oocyte cytosol, whereas only the 110-kDa R5020-binding protein was present in oocyte plasma membrane. A progesterone-binding protein (msPR) was identified in crude microsomal, rather than purified plasmalemmal, membranes from porcine liver (84,85). On solubilization, a moderate-affinity site with a dissociation constant (K_d) of 69 nM was found, but, after further purification, affinity decreased to K_d of 228 nM. The final fraction contained two novel peptides of 28 and 56 kDa. Expression of msPR-cDNA in CHO cells led to slightly increased progesterone binding in microsomes, and administration of an antibody against msPR reduced rapid progesterone-initiated Ca⁺⁺ increases in sperm (85). Whether this work represents the first successful cloning

and expression of a steroid receptor associated with cell membranes will have to await confirmation. However, Falkenstein et al. (85) suggest that the native plasma membrane PR may actually be an oligomeric protein complex of about 200 kDa, composed only in part by 28- and 56-kDa peptides.

Glucocorticoids, Aldosterone, and Vitamin D

Progress has been made in the isolation and characterization of plasma membrane receptors for glucocorticoids, aldosterone, and $1,25(\text{OH})_2\text{D}_3$, although at this writing, evidence of cloning of the cDNA for any of these proteins is lacking. The membrane glucocorticoid receptor (mGR) was purified from lymphoma cells by immunoaffinity binding with an MAb coupled to Sepharose-4B; the protein displayed properties similar to iGR (55). Scatchard analysis of mGR yielded a K_d of 239 nM and B_{max} of 384 fmol/mg of protein, representing a somewhat higher number of binding sites but a lower affinity than that of the iGR. Peptide maps revealed some sequences that were unique to the membrane form (55,56). Further data suggest that the mGR in lymphoma cells is a transcript variant of the iGR (56) (Table 2). Properties of the aldosterone membrane receptor have been analyzed by means of [^{125}I]-aldosterone photoaffinity labeling. The protein has an apparent molecular mass of 50 kDa and appears to be distinct from intracellular receptor (86).

The pursuit of membrane receptor for $1,25(\text{OH})_2\text{D}_3$ (pmVDR) by affinity isolation has been hampered by the fact that most ligand derivatives lack sufficient binding activity. Nevertheless, work by Baran et al. (87) indicates that the vitamin D analog, [^{14}C]- $1\alpha,25$ -dihydroxyvitamin D_3 bromoacetate, does exhibit a moderate degree of specific binding to a 36-kDa protein in plasma membranes of rat osteoblast-like cells. Using sequence determination and Western blot, the labeled membrane protein was identified as annexin II, part of a family of membrane-binding proteins previously implicated in the regulation of Ca^{++} signaling, tyrosine phosphorylation, and apoptosis. Partially purified plasma membrane proteins and purified annexin II exhibited specific and saturable binding for [^3H]- $1\alpha,25(\text{OH})_2\text{D}_3$, and antibodies to annexin II inhibited [^{14}C]- $1\alpha,25(\text{OH})_2\text{D}_3$ bromoacetate binding to plasma membranes and also inhibited hormone-induced increases in intracellular calcium in osteoblast-like cells. Hence, these initial results (87) suggest that annexin II may serve as a receptor for rapid actions of $1,25(\text{OH})_2\text{D}_3$ in rat osteoblast-like cells, but it is not known if this receptor system functions in other cell types. In independent studies, classic biochemical strategies, coupled with analyses of specific binding, were used to isolate the vitamin D membrane receptor (pmVDR) from intestinal epithelium of chicks (88). Basal-lateral membranes were solubilized with detergent and subjected to ion-exchange and gel filtration chromatography. Binding activity eluted with a protein of 65 kDa, with a K_d of 0.7 nM

(88). A highly specific antibody toward plasma membrane VDR failed to recognize the nuclear receptor in Western analyses. On the other hand, a commercially available MAb generated against the "classic" nuclear receptor reacted with many proteins in nuclear fractions of chick intestine, including a band that comigrated with authentic recombinant protein, but did not detect VDR in basolateral membranes (89). Antibody to the plasma membrane receptor, but not to the nuclear receptor, blocked hormonal activation of PKC. The 65-kDa protein was also observed to bind the affinity ligand, [^{14}C]- $1\alpha,25$ -dihydroxyvitamin D_3 bromoacetate, and labeling was diminished in the presence of excess nonradioactive ligand (89). Electron microscopic studies of duodena vascularly perfused with control media, $1,25(\text{OH})_2\text{D}_3$, or $24,25(\text{OH})_2\text{D}_3$ followed by immunochemical staining revealed that $1,25(\text{OH})_2\text{D}_3$, but not control media or $24,25(\text{OH})_2\text{D}_3$, resulted in dramatically enhanced nuclear localization of the putative membrane receptor (89).

Varied Forms of Steroid Hormone Receptors in Plasma Membranes

Collectively, current findings suggest that membrane receptors for steroid hormones are, in certain instances, transcriptional copies (estrogen) or variants (glucocorticoids) of nuclear receptors and, in other instances, products apparently unrelated to intracellular receptors (aldosterone and vitamin D). There is evidence for alternatively spliced transcripts of several steroid receptors, and these variant receptors give rise to proteins of different molecular size and, possibly, modified properties (56). Membrane insertion of receptors in primary transcript form would likely require one or more hydrophobic regions, and post-translational modification of receptor protein leading to cell membrane targeting may also occur, including phosphorylation, glycosylation, and addition of lipid anchors or other modifications, such as palmitoylation or myristoylation. Surface steroid hormone receptors may also be part of a multimeric complex including a "classic" nuclear receptor but bound to as-yet-unidentified transmembrane proteins and coupled to membrane-associated signaling molecules (3,7,15,79). Alternatively, plasma membrane receptors for steroids may have several common structural features with, but may be distinct from, the intracellular steroid hormone receptors (88,89). In the case of retinoic acid and estradiol, binding to known membrane proteins, such as M6P/IGF-2 receptor (75) or HER-2 receptor (90), respectively, may modulate some ligand effects. Progesterone appears to interact directly with oxytocin receptor, a G-linked protein at the cell surface, and inhibits some functional effects of oxytocin signaling, thus suppressing uterotonic activity of oxytocin (91). Progesterone congeners also bind with moderate affinity to γ -aminobutyrate type A (GABA_A) receptors that comprise ligand-gated ion channel complexes (4,28). Absence of the γ -subunit of GABA_A receptor in appropriate knockout mice results in a significant decrease in

sensitivity to neuroactive steroids such as pregnanolone (92). Similarly, acute vascular relaxation induced by pharmacologic levels of E_2 may be mediated by its binding to the regulatory subunit of Maxi-K channels in membranes (93), thus supporting the view that some effects of steroids, at least at high micromolar concentration, may be mediated by known membrane receptors with previously unrecognized steroid-binding sites.

Using ER α gene knockout (ERKO) mice, Gu et al. (94) showed that rapid actions of estradiol at 50 nM on kainate-induced currents in hippocampal neurons still occur, and the effect is not inhibited by ICI 182,780, a pure antagonist of hormone binding to both ER α and ER β . These investigators suggest that a distinct estrogen-binding site exists in neurons and appears to be coupled to kainate receptors by a cAMP-dependent process. However, it is important to note that alternatively spliced forms of ER α (95), as well as ER β (96), can occur in ERKO mice, thus complicating the interpretation of these results. Moreover, uterine tissues of ovariectomized ERKO mice exhibit 5–10% of the estradiol binding present in wild-type uteri (95,97), and the significance of these residual estrogen-binding sites in ERKO target cells is unclear. Nonetheless, further development of double ER α and ER β gene knockouts and perfection of this new technology should prove important in deciphering the contribution of “classic” and novel receptor forms in hormone action.

In future work, it will be important to pursue isolation and characterization of constituent proteins from homogeneous plasma membranes prepared in the presence of proteinase inhibitors (18,76,98). Verification of their purity should be confirmed by use of a balance sheet for enzyme or other membrane markers (18,76). Screening for activity of receptor would benefit from the use of independent approaches, such as ligand binding with radio- or photoaffinity-labeled steroids and immunoassay directed toward known intracellular receptors (15,31,55,78,86). These several approaches may detect membrane receptors originating from a transcript other than that of intracellular receptor. As with the mixed steroid hormone-binding protein systems known to occur within cells and in their extracellular fluids, it may well be that multiple forms of receptor proteins for steroids coexist in plasma membranes, thus complicating efforts to isolate and characterize the individual binding species in this cell compartment. Our efforts to understand ligand-receptor interactions are often limited by simplistic “lock-and-key” models that may not accurately reflect the true state of complex molecular signaling cascades. Study of the molecular organization of several neurotransmitter receptor families has already shown that extraordinary biologic variability occurs, with multiple “keys” and multiple “locks” sometimes involved in ligand-receptor recognition (99). We must consider the existence of similar high-affinity, but possibly multivalent and multifunctional, receptors in the steroid hormone superfamily (75,91–93).

Perspectives

Ever since the discovery of chromosomal puff induction by ecdysone, cell regulation by steroid hormones has focused primarily on a nuclear mechanism of action. However, even the venerable steroid hormone ecdysone elicits rapid membrane effects that may facilitate later nuclear alterations (100). Indeed, membrane-initiated responses appear to be the cell's earliest response to steroids and may be prerequisite to subsequent genomic responses (2,3,7,10; see also Fig. 1). Coupling of surface membrane, cytoplasmic, and nuclear responses may offer a progressive, ordered expansion of initial signal. Accordingly, the terms *genomic* and *nongenomic* may not accurately define such a response continuum (101). Future investigations should focus on potential interactions of membrane and nuclear steroid receptors that may promote activation of transcription and other specific hormonal responses. Molecular details of cross-communication between steroid and peptide receptors are also beginning to emerge (3,98), and membrane steroid receptors may be in a pivotal location to promote convergence among diverse signaling pathways (Fig. 1). Indeed, the consequences of steroid hormone recognition at the outer cell membrane of target, but not nontarget, cells are shared by numerous other classes of regulatory molecules (cf. ref. 102), including peptide hormones, neurotransmitters, drugs, plant lectins, mitogens, and antibodies (3). Although the agonists are manifold, the signaling mechanisms are few. Primary signal recognition at the surface would be fleeting, but the mutual specificities and affinities are high, and thus sufficient for setting the appropriate signal transduction chain in motion. However, until the current surge of renewed focus on this problem, identification of these instantaneous triggering interactions for steroid hormones has accumulated relatively slowly, having been limited by technical and microanalytic barriers that are now being surmounted.

Ligand-receptor interactions depend on an extensive array of extracellular and intracellular partners to localize to membrane microdomains, recruit signaling molecules, and trigger intracellular signaling pathways. As the consequences of surface interactions are analyzed in greater depth, it will be important to evaluate further the biologic role of rapid internalization of steroid-binding sites from plasma membranes via endocytotic-lysosomal pathways (2,3,88,101,103–105). These membrane-initiated events may involve cytostructural elements or scaffold proteins that contribute to signal propagation to the nucleus and the nuclear-protein matrix (2,101,104–107; Fig. 1). Thus, antibodies specific to intestinal membrane VDR reveal a vitamin D-induced redistribution of membrane receptor, a protein that appears distinct from intracellular receptor, to the nucleus within 5 min of binding ligand (89). It is unknown whether the membrane receptor has inherent DNA- or coregulator-binding capacity to alter transcription; alter-

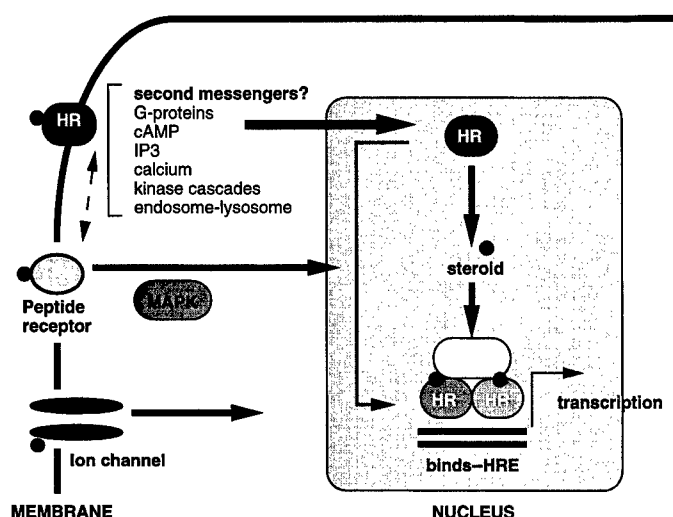


Fig. 1. Postulated mechanism of action of steroid hormones (black circles) in target cells with steroid hormone receptor (HR). In most current models, steroid binding to HR is believed to promote alterations in receptor conformation favoring enhanced association with coactivator proteins and with specific hormone-responsive elements (HRE) in the nucleus, leading, in turn, to initiation of selective gene transcription. However, the latter model fails to account for numerous, rapid cell responses to steroid treatment (see Table 1 and text). These deficiencies in the genomic model of hormone action require integration with the latter observations. In the model shown here, steroids may also bind to a membrane HR, with potential for promotion of hormonal responses via a complementary pathway that may cross-communicate or interact directly with the genomic mechanism. As noted in the text, membrane HR may be known molecules (kinases, ion channels, other receptors) with previously unrecognized binding sites for steroid, new isoforms of HR in membranes, "classic" forms of HR complexed with other membrane-associated proteins, truly novel membrane proteins, or a combination of these. Available evidence indicates that liganded membrane HR may affect one or more of several pathways, including modulation of ion channels, leading to enhanced flux of ions, notably Ca^{++} ; interaction with peptide membrane receptors; and activation of G-proteins, nucleotide cyclases, and MAPK, with resultant increases in their catalytic products (see Table 1). These membrane interactions may promote phosphorylation of HR itself via steroid-induced or ligand-independent pathways. The intricate array of physiologic responses of cells to steroid hormones may occur as a consequence of a synergistic feed-forward circuit in which steroids activate cell membrane signaling pathways that act, in turn, to enhance the transcriptional activity of HR (Table 1). Active reconsideration of the unqualified genomic model of nuclear receptor action is ongoing, and the probable importance of alternate signaling pathways elicited by surface recognition is now increasingly evident.

natively, it could serve to shuttle ligand to the nuclear-localized fraction of receptor. As has frequently been noted from these laboratories (cf. ref. 105), the cellular mechanisms governing the further transport and targeting of signaling molecules are powerful avenues of current investigation.

Many issues remain to be resolved for fuller understanding of the biologic actions of steroid hormones. Foremost among these is the structural characterization of membrane

steroid hormone receptors. It is now abundantly clear that the nuclear receptor-mediated mechanism as the sole means by which steroid hormones act is incomplete (2,3,5,7,15,107). It is likewise unmistakable that membrane effects of steroid hormones represent an established phenomenon that is by no means to be construed as alternative to the genomic pathway, and that demands continued investigation. Indeed, the chain of membrane-initiated events is helping to account for the relatively prolonged, apparent silence between the capture of the hormone at the surface of its preferential target and the eventual outcome in augmented genomic activities. In challenging the dogma that steroid hormones act exclusively via intracellular receptors, the membrane receptor experiments reviewed here provide a persuasive paradigm for a potentially new class of drugs for human therapy. The clinical use of steroid hormone agonists and antagonists has substantially changed the course of many hormone-related diseases, but side effects of many agents currently in use are also significant. In-depth analysis of the relative contributions of nuclear and membrane-initiated activities in steroid receptor biology may lead to the development of pharmaceutical agents that exert differential activities in the two pathways, thus favoring more selective drug delivery and promoting the emergence of novel approaches for treatment of many cell metabolic and proliferative defects.

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Epidermal Growth Factor Receptor and Tyrosine Phosphorylation of Estrogen Receptor

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Activation of estrogen receptor- α (ER α) by growth factors in the absence of estrogen is a well-documented phenomenon. To study further this process of ligand-independent receptor activation, COS-7 cells without ER were transfected with both ER and epidermal growth factor receptor (EGFR). In the absence of estrogen, epidermal growth factor (EGF) stimulated rapid tyrosine phosphorylation of ER in transfected COS-7 cells. Similarly, in MCF-7 breast cancer cells that have natural expression of ER and EGFR, EGF promoted acute phosphorylation of serine and tyrosine residues in ER, and a direct interaction between ER and EGFR after treatment with EGF was found. In confirmation of a direct interaction between ER and EGFR, activation of affinity-purified EGFR tyrosine kinase *in vitro* stimulated the phosphorylation of recombinant ER. The cross-communication between EGFR and ER appears to promote significant stimulation of cell proliferation and a reduction in the apoptotic loss of those cells that express both receptor signaling pathways. However, COS-7 cells transfected with both ER and EGFR show minimal stimulation of classical estrogen response element (ERE)-dependent transcriptional activity after stimulation by EGF ligand. This suggests that the proliferative and antiapoptotic activity of EGF-induced ER activation may be dissociated from ERE-dependent transcriptional activity of the ER.

Key Words: Epidermal growth factor; estrogen receptor; tyrosine phosphorylation; estradiol; MCF-7 cells; apoptosis.

Introduction

The estrogen receptor (ER) is a member of a large family of nuclear receptors that share a common structural and functional organization. These receptors are generally considered to function as ligand-activated transcription factors

(1–3). However, accumulating evidence has demonstrated significant cross-communication between steroid hormone receptors and peptide growth factor signaling pathways, with some reports suggesting that growth factors may promote activation of steroid receptors even in the absence of natural ligand. Agents capable of exerting such ligand-independent activation of ER include epidermal growth factor (EGF) (4–9), transforming growth factor- α (7), heregulin (10), insulin (11), insulin-like growth factor-1 (7,8,12–14), and dopamine (15). Under estrogen-free conditions, *in vivo* administration of EGF alone mimics the effects of estrogen in the mouse reproductive tract (16,17). In mice lacking ER- α expression, both estrogen- and EGF-stimulated uterine growth is blocked (17). Thus, ER may mediate the transcription of target genes by integrating signals from growth factor-activated pathways as well as from steroid hormone binding (18).

It is notable that cooperative interactions between erb B and nuclear receptors were first reported more than a decade ago (19). The EGF receptor (EGFR) is a 170-kDa transmembrane glycoprotein that consists of an extracellular ligand-binding domain in its amino terminus, a transmembrane-spanning region, and a cytoplasmic EGF-stimulated protein tyrosine kinase in its C-terminus. EGFR is part of the erb B family of growth factor receptors. On ligand binding and dimerization, the receptor undergoes phosphorylation on tyrosine residues. EGFR activation results, in turn, in the phosphorylation of downstream protein kinases and the subsequent activation of specific transcription factors. With emerging evidence for estrogen-stimulated activation of mitogen-activated protein kinase (MAPK) signaling pathways (8), growth factor- and steroid hormone-dependent mitogenic cascades may well have significant interactions.

The ER is characterized by six major functional domains often termed A–F. The A/B region contains an N-terminal transactivation domain, AF-1; the C region harbors the DNA-binding domain, while the D-region is involved in nuclear localization signaling; and E/F contains the C-terminal portion of the receptor and is involved in hormone binding, dimerization, and the function of a second transactivation domain, AF-2 (2,3,20). AF-1 and AF-2 appear to contribute synergistically to the transcription of ER-regulated target genes, but they have different mechanisms of activation. AF-1 activity is highly dependent on serine phosphorylation by MAPK signaling (8), while AF-2 is activated

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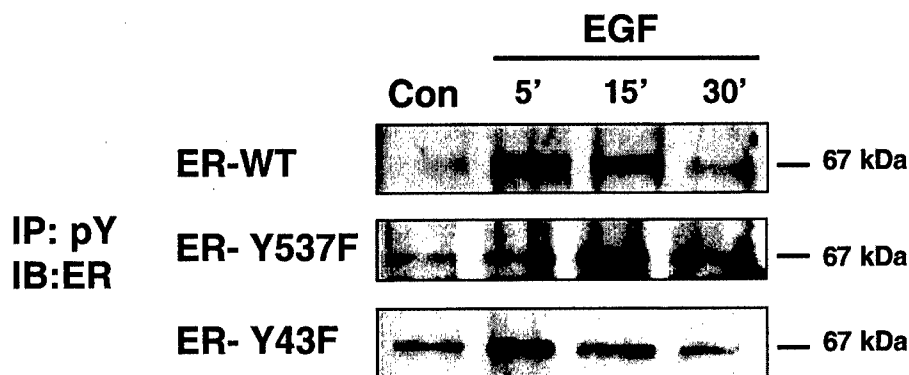


Fig. 1. EGF treatment promotes tyrosine phosphorylation of ER. COS-7 cells were transfected with EGFR and ER vectors and then treated with control vehicle (Con) or 2 nM EGF for 5, 15, and 30 min. Cell lysates were processed as described in Materials and Methods, then immunoprecipitated (IP) using an antiphosphotyrosine antibody (pY), before electrophoresis and immunoblotting (IB) with anti-ER antibody (ER). Treatment groups included COS-7 cells transfected with EGFR + ER-wild type (WT), EGFR + ER-Y537F mutant (Y537F), and EGFR + ER-Y43F mutant (Y43F). A representative blot from one of three experiments is shown.

by binding estrogenic ligands. EGF-stimulated activation of ER may be mediated, in part, by the AF-1 domain of ER. Within the AF-1 domain, phosphorylation of serine-118 appears to be required for full activity of AF-1, and this phosphorylation step is mediated by MAPK (8,9,21). Additional phosphorylation sites in ER that may participate in the transcriptional activation of ER include serine-167, a major estradiol-induced phosphorylation site on ER (22), as well as serine-104 and serine-106 (23).

Several reports have also provided evidence for significant phosphorylation of the ER at tyrosine residues (10, 24–26). Although a number of initial studies suggested that phosphorylation of ER at tyrosine-537 (Y537) may be important for DNA binding and for transcriptional activation (25–28), more recent evidence indicates that phosphorylation at Y537 of ER is not an absolute requirement for hormone binding to ER or for activation of ER-dependent transcription (29,30). However, the role of ER tyrosine phosphorylation sites in the regulation of cell proliferation and in the cellular response to growth factor stimulation (24,31) has not been fully evaluated.

To assess the hypothesis that EGF-mediated activation of ER may involve tyrosine phosphorylation of ER, we used several different experimental approaches to evaluate cross-communication between ER and EGFR. The combined results suggest that EGFR tyrosine kinase interacts directly with ER in solution and in intact cells, leading to tyrosine phosphorylation of ER. This alteration in ER may then contribute to the promotion of estrogen-independent activation of ER-mediated transcription and cell proliferation.

Results

EGF Treatment Promotes EGFR-Mediated Tyrosine Phosphorylation of ER in Intact Cells

Previous work has demonstrated that ER can undergo tyrosine phosphorylation in a process that appears to be mediated by cellular tyrosine kinase receptors (10,24–26,

30). To determine whether tyrosine phosphorylation of ER can be mediated by EGFR, COS-7 monkey kidney cells with low to nil EGFR and no ER were transiently transfected with expression vectors for EGFR and ER-wild type and then treated, in the absence of estrogen, with 2 nM EGF. The results showed that ER-wild type is tyrosine phosphorylated after cell stimulation with EGF in the absence of estrogen (Fig. 1). The level of ER phosphorylation increased significantly by 5 min and then declined after 30 min. To assess the contribution of tyrosine-537 in ER in this process, COS-7 cells were next transfected with EGFR and ER with directed mutation of tyrosine-537 to phenylalanine (Y537F). The mutated ER-Y537F showed a modest increase in basal levels of ER phosphorylation (Fig. 1). In addition, cells transfected with ER-Y537F exhibited a reduction in the level of receptor phosphorylation at 5 min after EGF treatment but no apparent decrease at later times (Fig. 1). This result suggests that this is not the tyrosine residue that is primarily phosphorylated in ER or that more than one tyrosine residue in ER may be phosphorylated (30). To evaluate the potential role of other tyrosine residues in ER, COS-7 cells were transfected with EGFR and ER with a directed mutation of tyrosine-43 to phenylalanine (Y43F). This alteration elicited an increase in the basal level of tyrosine phosphorylation of ER. In addition, the EGF response of COS-7 cells containing EGFR and ER-Y43F appeared more deficient, especially when compared with control (Fig. 1). These findings may indicate that tyrosine residues other than the 537-residue may participate in EGFR-mediated phosphorylation of ER.

EGF Stimulates Low Levels of Estrogen Response Element-Dependent Transactivational Activity of ER in Absence of Estrogen

The effects of EGF and estrogen on transcriptional activation of an estrogen response element (ERE) were assessed using a reporter plasmid, pERE-BLCAT, containing the vitellogenin A2 ERE (32). COS-7 cells were transfected with

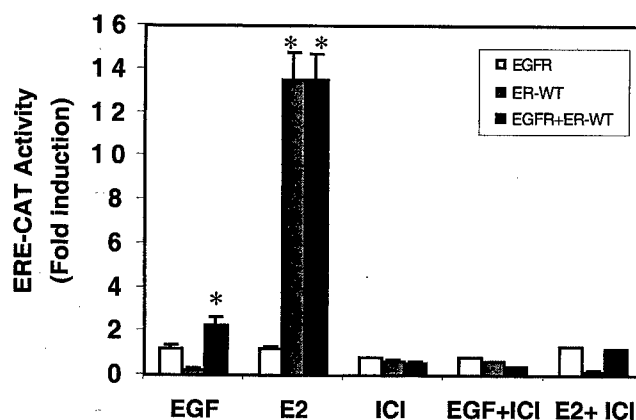


Fig. 2. EGF stimulates a low level of transactivation activity of ER in the absence of estrogen ligand. COS-7 cells were transfected with EGFR and pERE-BLCAT reporter gene (EGFR); ER and pERE-BLCAT (ER-WT); or EGFR, ER, and pERE-BLCAT (EGFR + ER-WT). Cells were treated with control vehicle, 2 nM EGF, 10 nM estradiol 17- β (E_2), 1 μ M ICI 182,780 (ICI), or combinations of these reagents for 18 h. After treatment, cell lysates were prepared and analyzed for ERE-CAT activity by established methods. Transactivation of the CAT reporter gene is expressed as fold induction of the untreated control. Each bar represents the mean \pm SE of determinations from three individual experiments. Asterisks denote results significantly different from control at $p < 0.05$.

ERE-chloramphenicol acetyltransferase (CAT) reporter gene in combination with either EGFR alone or EGFR plus ER-wild type (ER-wt). Treatment with estradiol-17 β induced transactivation of the ERE-CAT reporter in cells transfected with ER-wt by about 14-fold ($p < 0.001$) (Fig. 2). By contrast, treatment with EGF elicited ER transactivation by only about two-fold ($p < 0.05$) in cells transfected with ER-wt and not at all in those cells transfected with EGFR alone. Of importance, ER transactivation induced by estradiol and by EGF were both inhibited by coadministration of the pure antiestrogen ICI 182,780 (33), thus suggesting that these activities are mediated by ER.

EGF Treatment Promotes Interactions

Between EGFR and ER in Human Breast Cancer Cells

To assess the potential direct interaction between EGFR tyrosine kinase and naturally expressed ER in intact cells, MCF-7 human breast cancer cells known to express significant levels of EGFR (34) were treated with 2 nM EGF for 1–60 min in vitro. Thereafter, the cells were disrupted and processed for immunoprecipitation with anti-EGFR antibodies and then immunoblotting with anti-ER antibodies (Fig. 3A). The results showed an enhanced interaction between ER and EGFR that was evident by 1 min after EGF treatment, followed by a peak at 15–30 min and then a decline to baseline levels of receptor association by 60 min (Fig. 3A). As an additional control, the treated membrane was stripped and reprobed using anti-EGFR antibody to confirm that EGFR did not significantly vary during the course of the experiment (Fig. 3B). The time course of the direct interaction between ER and EGFR was compared with the known phosphorylation of serine residues in ER (Fig. 3C) and the phosphorylation of tyrosine residues in ER (Fig. 3C) after treatment of MCF-7 cells with 2 nM EGF in vitro.

EGF Stimulation of EGFR

Promotes Phosphorylation of ER in Solution

To assess further the interaction of EGFR tyrosine kinase with ER, these proteins were studied in solution in vitro. It is notable that EGF stimulation of immunoaffinity-purified EGFR kinase activity induces a significant increase in EGFR autophosphorylation (35–37), a phenomenon observed in the present experiment (Fig. 4). Incubation of the affinity-purified human EGFR with purified recombinant human ER in the presence of estrogen and EGF induced significant phosphorylation of ER in the absence of any other cellular kinase enzymes in solution (Fig. 4). The level of ER phosphorylation was substantially higher than that found in the absence of EGFR. The added phosphorylation is likely owing to derivatization of tyrosine residues in ER by the action of EGFR tyrosine kinase.

EGF-Induced Cell Proliferation

is Enhanced and Cell Death is Reduced When Both EGFR and ER Are Present

EGF (38–40) and estrogen (41) are both known mitogens for breast cancer cells. To assess the potential contribution of EGFR signaling pathways in ER-mediated cell growth, COS-7 cells were transiently transfected with either control vectors, EGFR vector alone, ER-wt vector alone, or both receptor vectors. Under these conditions, treatment with EGF elicited no significant stimulation of the growth of parental or mock-transfected COS-7 cells, nor COS-7 cells transfected only with ER-wt ($p > 0.05$) (Fig. 5A). By contrast, EGF markedly enhanced the growth of EGFR-transfected COS-7 cells to about 1.6 times that of controls ($p < 0.05$) (Fig. 5A). Cell proliferation induced by EGF was further enhanced to about 2.1 times that of controls when both ER-wt receptors and EGFR were cotransfected in COS-7 cells ($p < 0.01$) (Fig. 5A). A modest reduction in

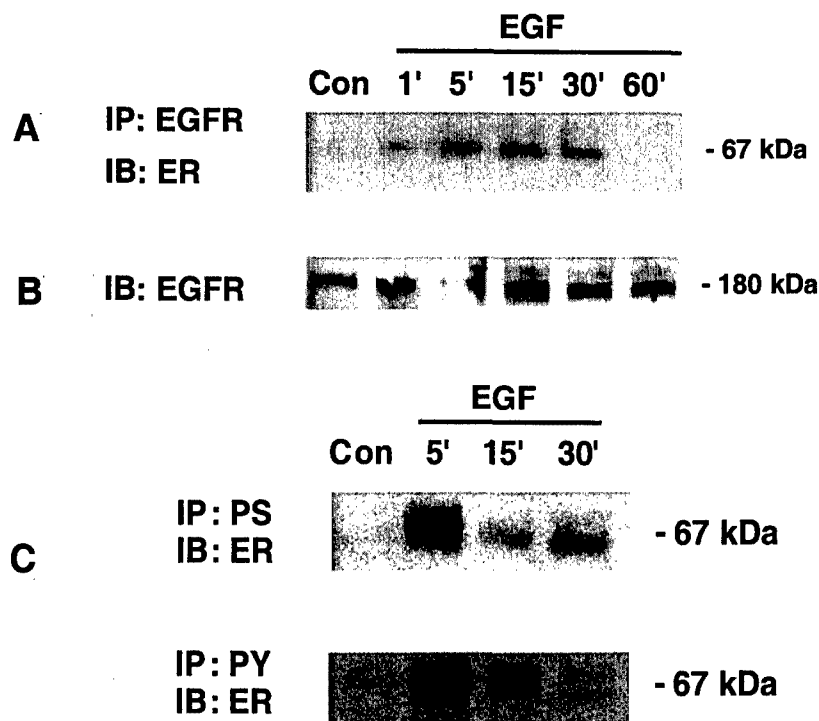
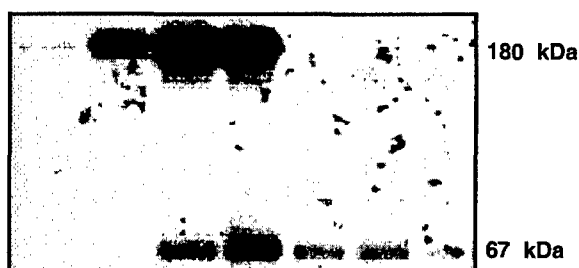


Fig. 3. EGF treatment of MCF-7 human breast cancer cells promotes association of EGFR with ER and stimulation of ER phosphorylation. (A) EGF treatment promotes association of EGFR with ER in MCF-7 cells. MCF-7 cells were treated with control vehicle (Con) or 2 nM EGF for 1, 5, 15, and 30 min. Cell lysates were prepared and processed as described in Materials and Methods. Immunoprecipitation (IP) was done using anti-EGFR antibody before electrophoresis, and immunoblotting (IB) was done with anti-ER antibody. A representative blot from one of six experiments is shown here. (B) EGFR in MCF-7 cells. As an additional control experiment, treated membrane from panel (A) was stripped and reprobed with anti-EGFR antibody to ensure no significant variation in EGFR during the course of the treatment. (C) EGF treatment promotes phosphorylation of serine and tyrosine residues in ER. MCF-7 cells were treated with control vehicle (Con) or 2 nM EGF for 5, 15, and 30 min. Cell lysates were prepared and processed as described in Materials and Methods. IP was done using either antiphosphoserine (PS) or antiphosphotyrosine (PY) antibody before electrophoresis, and IB was done with anti-ER antibody.



EGF	-	+	+	+	+	-	-
EGFR	+	+	+	+	-	-	-
E ₂ β	+	+	-	+	+	+	-
ER	-	-	+	+	+	+	+

Fig. 4. Phosphorylation of purified recombinant ER in vitro by activated affinity-purified EGFR tyrosine kinase. ER, EGFR, or both receptor proteins in the presence of 100 nM estradiol 17-β (E₂), 100 nM EGF, or both ligands in solution were incubated in vitro. After the addition of 10 μM ATP and 1 μCi (6000 Ci/mmol) of [γ-³²P]-ATP, samples were incubated at 5°C for 15 min. Proteins were separated on 7.5% SDS-PAGE gels, and after running, gels were dried and exposed for autoradiographic analysis using established methods. A representative film from three experiments is shown.

the anticipated level of EGF-stimulated cell growth occurred when COS-7 cells were transfected with EGFR in combination with ER isoforms mutated at tyrosine-537 ($p > 0.05$) (see Fig. 5A). Moreover, COS-7 cells transfected with EGFR and ER forms mutated at tyrosine-537 showed significantly less proliferation in response to EGF stimulation than those cells containing a combination of ER-wt receptors and EGFRs ($p < 0.05$).

Since cumulative cell growth is a function of both cell proliferation and cell loss (42–44), EGF-induced inhibition of cell death was also assessed using a modified TdT-mediated dUTP nick-end labeling (TUNEL) assay (45) in COS-7 cells grown in vitro under growth factor-depleted conditions (Fig. 5B). The cells were first plated in standard media for 48 h, and then the media were changed to phenol-red free media containing 0.1% dextran-coated, charcoal-treated fetal bovine serum (DCC-FBS) to promote estrogen-free and serum-depleted conditions. EGF-induced blockade of apoptosis was assessed in COS-7 cells in the native state or transfected with control vector, EGFR, EGFR and ER-wt, EGFR and ER-Y537F mutant, or EGFR and ER-Y537A mutant. After transfection, cells were treated with 10 nM EGF and cultivated 72 h before TUNEL assay,

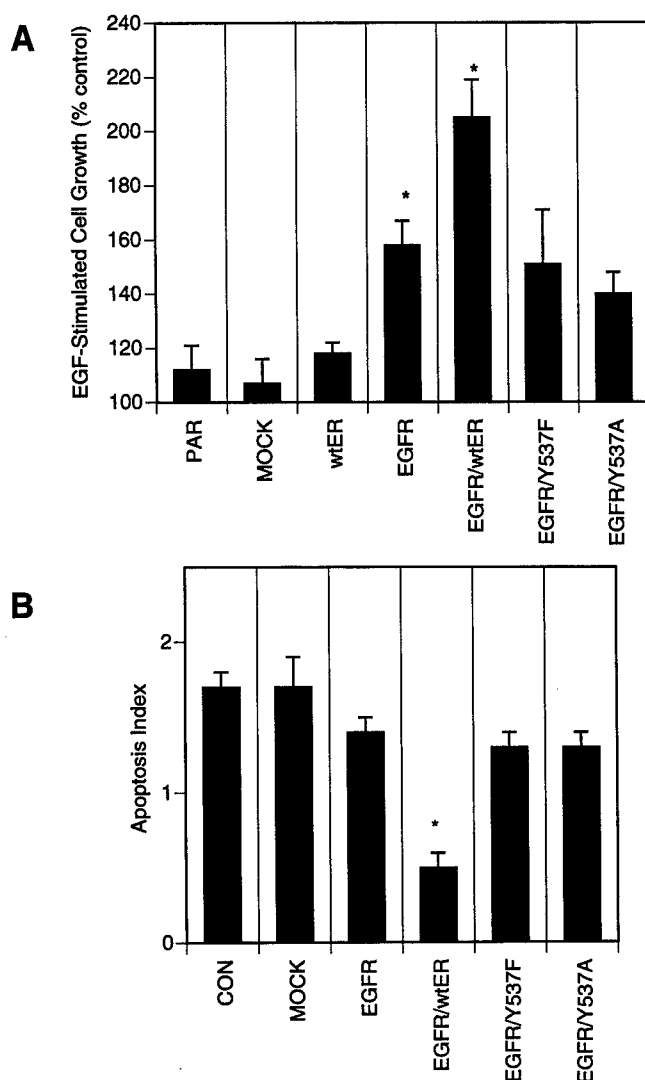


Fig. 5. EGF treatment stimulates enhanced proliferation and reduced apoptosis of COS-7 cells transfected with EGFR and ER. **(A)** EGF-induced cell growth was assessed in COS-7 cells in the native state (PAR) or transfected with control vector (MOCK), ER-wild type (wtER), ER-Y537F mutant (Y537F), ER-Y537A mutant (Y537A), EGFR, or combinations of the receptor vectors. After transfection, cells were treated with control vehicle alone or 10 nM EGF. Cells were then cultivated further, and final cell numbers were quantitated after 72 h for each treatment group as indicated. Data (mean \pm SE) were collected from 10 to 20 independent experiments. **(B)** EGF-induced inhibition of cell death was assessed using a modified TUNEL assay (45) in COS-7 cells. The cells were first plated in standard medium for 48 h, and then the medium was changed to phenol-red free D-MEM or RPMI containing 0.1% DCC-FBS to promote estrogen-free and serum-depleted conditions. EGF-mediated reduction of apoptosis induced by serum depletion was assessed in COS-7 cells in the native state (CON) or transfected with control vector (MOCK), EGFR, EGFR and ER-wild type (EGFR/wtER), EGFR and ER-Y537F mutant (EGFR/Y537F), or EGFR and ER-Y537A mutant (EGFR/Y537A). After transfection, cells were treated with 10 nM EGF and cultivated for 72 h before the TUNEL assay, with calculation of the apoptosis index as before (45). Data (mean \pm SE) were collected from four to six independent experiments.

with calculation of the apoptosis index as before (45). The results showed that cells transfected with EGFR and ER-wt, but not EGFR and ER forms mutated at tyrosine-537, had a reduced level of apoptosis as compared with appropriate controls ($p < 0.01$) (Fig. 5B).

Discussion

The activation of ER by growth factors in the absence of estrogen is a well-documented phenomenon and may play a critical role in steroid receptor signaling and breast cancer development (8,10,17,42,46). The present study provides evidence for direct cross-communication between EGFR tyrosine kinase and ER and suggests that such interactions between growth factor receptors and steroid receptors may contribute to the modulation of hormone activity in a ligand-independent manner. The current findings add to a growing body of evidence that the classic ER can participate in the activation of transcription and cell proliferation by different cellular pathways.

Phosphorylation of ER at serine and tyrosine residues appears to contribute to receptor activation and, possibly, binding to DNA (2,11,22,25,26,29,30,47). MAPK-mediated phosphorylation of serine residues plays a role in the activation of AF-1 in the absence of estrogen. However, to obtain full activation of the AF-1 domain, it appears that other residues, as yet undetermined, must also be phosphorylated (8). Our results show that, after EGF stimulation, ER can be phosphorylated on tyrosine residues and more than one tyrosine may be phosphorylated. Site-directed mutation of ER tyrosine residues at positions 43 and 537 appears to enhance basal levels of ER tyrosine phosphorylation and promotes alterations in the time course and the level of ER tyrosine phosphorylation after treatment with EGF. Similarly, previous data have demonstrated tyrosine phosphorylation of ER after stimulation of tyrosine kinase signaling in MCF-7 cells by heregulin, a ligand for HER-1/HER-2/HER-3 receptors (10). It remains to be determined what contribution tyrosine phosphorylation may make in regulating the activation of AF-1 or the interactions between AF-1 and AF-2 domains of ER.

In the present studies, EGF significantly enhanced the growth and reduced the apoptotic loss of ER-negative COS-7 cells after transfection of ER in monkey kidney cells. Under estrogen-free conditions, *in vivo* administration of EGF similarly mimics the growth-promoting effects of estrogen in the mouse reproductive tract (16,17). In addition, in knockout mice lacking ER- α , both estrogen- and EGF-stimulated uterine growth is blocked, suggesting the importance of ER for the promotion of EGF-mediated growth (17). However, with assays of ER transcriptional activity using an ERE-CAT reporter gene, the present studies demonstrated that ER is only minimally activated by EGF in the absence of estrogen, a result consistent with many earlier reports (2,4-9) but contrasting, in part, with one study (48).

Although EGF promotes significant proliferation of cells containing ER, it does not stimulate a large increment in ERE-dependent transcription. This finding is a paradox. However, results from several recent studies suggest that cell growth and ERE-dependent transcription may not be associated. Kousteni et al. (36) have reported that the antiapoptotic action of estrogen in target cells can be dissociated from the transcriptional activity of the classic receptor, and our results appear to support this finding. Remarkably, estrogen-dependent gene transcription can be inhibited by nitric oxide, but DNA synthesis induced by estradiol is unaffected by nitric oxide, thus suggesting again that some effects of estradiol are mediated by a pathway that is not dependent on ERE-related transcription (49). A discordance between ERE-dependent transcriptional activity and estrogen-dependent proliferation also led earlier investigators to propose that the two processes may be exclusive cell functions (50). Collectively, these findings are consistent with the hypothesis that ER-dependent proliferation and inhibition of apoptosis may occur along a different pathway than ERE-dependent transcription (see also ref. 51). Further studies will now be required to test this hypothesis.

Cross-communication between peptide growth factor pathways and ER may prove to be very important in modulating hormonal activity in normal and aberrant tissue. One potential cellular site for interaction between ER and EGFR may be caveolae, specialized microdomains in plasma membrane. Caveolae are thought to occur in most cell types (52), although with reduced expression in breast cancer cells (53). Caveolae are enriched in EGFR, and EGF treatment promotes the recruitment of multiple signaling molecules to caveolae (52,54). A portion of ERs in target cells also localizes in caveolar membrane fractions (31,55,56), and ER can interact with caveolin-1, a defining protein in caveolae that provides a scaffold for the assembly of signaling molecules (57).

A number of studies have now documented that ER is subject to phosphorylation and activation by several peptide growth factors with consequent ERE-mediated gene expression (5–7,12,15,58). Altered elements in growth factor signaling pathways, such as receptor amplification and/or overexpression, may directly influence steroid hormone action in human breast cancers (46). One major problem in breast cancer management is the conversion of estrogen-sensitive to hormone-resistant malignancies after initiation of antiestrogen therapy (59). The molecular basis for this hormone-independent progression of breast cancer is not clear. However, enhanced cross-communication between growth factor receptor pathways and ER during cancer progression could contribute to ER activation in the absence of hormone. This development could then result in a reduced response to antiestrogens (46). Current findings indicate that EGFR plays a leading role in the progression of breast tumors (38). In patients with breast cancer, prognosis is inversely correlated with overexpression and/or amplifica-

tion of EGFR. In addition, an inverse correlation in the expression of ER and EGFR in breast cancers correlates with aggressiveness of the disease and with the response to endocrine treatment (46). Of special significance in human breast cancer, increased signaling through the EGFR pathway also results from overexpression of HER-2, an important signaling partner for EGFR (60). It is hoped that further delineation of these complex pathways in breast cancer cells will lead to the design of novel therapies that combine antigrowth factor signaling strategies with antihormone measures.

Materials and Methods

EGF and estradiol-17 β were from Sigma (St. Louis, MO). ICI 182,780 (7 α -[9-(4,4,5,5,5-pentafluoropentylsulfinyl) nonyl] estra-1,3,5(10)-triene-3,17 β -diol), a compound with pure estrogen antagonist activity in vivo and in MCF-7 cells in vitro (33), was generously provided by Dr. Alan Wakeling (Astra Zeneca Pharmaceuticals). ER- α is a recombinant human protein (66 kDa) purified from a baculovirus expression system (PanVera, Madison, WI). The translated sequence, corresponding to Genebank entry M12674, is functionally active and binds estradiol with high affinity and high specific binding activity exceeding 5000 pmol of [³H]-estradiol bound/mg receptor protein (PanVera) (61–63), a finding confirmed in our laboratory (data not shown). EGFR (HER1) is purified from human carcinoma A431 cells by affinity chromatography methods (37). One unit of EGFR protein transfers 1 pmol of [³²P]-phosphate to angiotensin-II/min at 30°C at pH 7.4 (64) (PanVera). [⁸⁻³²P]ATP was from Perkin-Elmer (Boston, MA). Antibodies to ER and EGFR were from Oncogene Research (Cambridge, MA). Agarose-conjugated antiphosphotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY), and agarose-conjugated antiphosphoserine antibody (65) was from Sigma. Anti-EGFR agarose conjugate antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

COS-7 monkey kidney cells and MCF-7 human breast cancer cells (American Type Culture Collection [ATCC] Rockville, MD) were routinely maintained as before (10) in Dulbecco's modified Eagle's medium (D-MEM) and RPMI-1640 containing 10% FBS, 100 U of penicillin/mL, 100 μ g of streptomycin/mL, 25 μ g of gentamycin/mL, and 2 mM L-glutamine. At 48 h before each experiment, the medium was changed to phenol red-free D-MEM or RPMI-1640 containing 1% DCC-FBS (66) to promote estrogen-free conditions.

Plasmids

The plasmid, pEV7-HER1, was a gift from Dr. Ke Zhang (Amgen, Thousand Oaks, CA) (67). A reporter plasmid containing a palindromic ERE and the CAT gene, termed

pERE_BLCAT, was a gift from Dr. Malcolm Parker (Imperial Cancer Research Fund, London, UK) (10). In brief, an oligonucleotide sequence corresponding to an ERE derived from the vitellogenin A2 promoter of *Xenopus laevis* (−331 to −295) was cloned into the *Xba*I site of pBLCAT2.

The ER expression vectors used are derivatives of pIC-ER-F (68) and were obtained from ATCC. Site-directed mutations of ERs were constructed by established methods (27,69,70) using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The following primers were used:

1. ERY537F-P1: 5' AAGAACGTGGTGGCCCTCTTTGA CCTGCTGCTGGAGATG 3'.
2. ERY537F-P2: 5' CATCTCCAGCAGCAGGTCAAAGAG GGGCACCACGTTCTT 3'.
3. ERY43F-P1: 5' CCCCTGGGCGAGGTGTTTCTGGACA GCAGCAAG 3'.
4. ERY43F-P2: 5' CTTGCTGCTGTCCAGAAACACCTCG CCCAGGGG 3'.
5. ERY537A-P1: 5' AAGAACGTGGTGGCCCTCGCTGAC CTGCTGCTGGAGATG 3'.
6. ERY537A-P2: 5' CATCTCCAGCAGCAGGTCTAGCGAG GGGCACCACGTTCTT 3'.

Following site-directed mutagenesis, the ER cDNAs were excised from pIC-ER-F using *Eco*RI and ligated into the *Eco*RI site of the pCDNA₃ (Clontech, Palo Alto, CA). Restriction enzyme digestion was used to verify directional cloning. The following vectors were obtained: pCDNA₃ ER-WT, pCDNA₃ER-Y537F, pCDNA₃ER-Y537A, and pCDNA₃ER-Y43F.

Immunoprecipitation and Western Blots

Cells were grown in 100-mm Petri dishes and maintained in phenol red-free D-MEM, containing 1% DCC-FBS for 48 h. Cell transfections were carried out with methods as before (10) using 40 µg of Plus Reagent, 25 µL of Lipofectamine, 2 µg of pEV7-HER1, and 2 µg of either pCDNA₃ ER-WT, pCDNA₃ER-Y537F, or pCDNA₃ER-Y43F per plate. At 24 h after transfection, cells were treated with 2 nM EGF for different time periods. After treatment, cells were immediately washed 3 times with cold PBS and homogenized in cold mild lysis buffer (20 mM Tris-HCl, pH 8.0; 137 mM NaCl, 10% glycerol; 1% Triton X-100; 20 mM EDTA) in the presence of 1 µg/mL of leupeptin, 1 µg/mL of aprotinin, 50 µg/mL of trypsin inhibitor, 0.4 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1 mM sodium orthovanadate. Proteins were quantified using the BCA-200 Protein Assay Kit (Pierce, Rockford, IL). Employing methods as before (10), immunoprecipitation was done using 500 µg of total protein and 10 µL of antiphosphotyrosine agarose-conjugated antibody (clone 4G10; Upstate Biotechnology) or 2 µg/mL of anti-EGFR agarose-conjugated antibody (R-1, against receptor cell surface epitope; Santa Cruz Biotechnology), overnight at 4°C. After wash-

ing four times with mild lysis buffer, samples were resuspended in 2X Laemmli sample buffer, boiled for 5 min, and separated on 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. After transfer, nitrocellulose membranes were subjected to immunodetection with 1 µg/mL of anti-ER monoclonal antibody (clone TE-111, directed against amino acids 302–595 of ER-α) using the electrochemiluminescence Western blotting system according to the manufacturer's recommendations (Amersham Pharmacia, Arlington Heights, IL) (10).

In Vitro Phosphorylation

Studies of in vitro phosphorylation were conducted by a modification of established methods (26). In brief, a concentration of 15 pmol of EGFR, purified by affinity chromatography from human carcinoma A431 cells (37), was incubated in buffer containing 20 mM HEPES (pH 7.4) and 1 mM sodium orthovanadate with or without 100 nM EGF for 3 min at 30°C. Tubes were immediately transferred to ice and incubated for 5 min after the addition of recombinant ER-α (3.2 pmol) and 0.3% Triton X-100, in the presence or absence of 100 nM estradiol-17β. Then, a mixture of 4 mM MgCl₂, 2 mM MnCl₂, 10 µM adenosine triphosphate (ATP), and 1 µCi (6000 Ci/mmol) [γ-³²P]-ATP (NEN, Boston, MA) was added, and samples were incubated for 15 min. Reactions were terminated by the addition of 2X Laemmli sample buffer and boiled at 100°C for 5 min. Proteins were separated on 7.5% SDS-PAGE gels, and after running, gels were dried and exposed for autoradiographic analysis.

CAT Reporter Gene Assays

In selected experiments, ER transcriptional activity was assessed with an ERE-CAT reporter gene. Transient transfections were performed with methods as before using the pERE-BLCAT reporter vector (10,27). In brief, cells in 60-mm Petri dishes were transfected using 2 µg of pERE-BLCAT vector and 1.5 µg pEV7-HER1 in combination with 1.5 µg of pCDNA₃ or 1.5 µg of pEV7-HER1 in combination with 1.5 µg of pCDNA₃ER-WT. Then, 30 µL of Superfect reagent (Qiagen, Valencia, CA) were added per dish. Transfection was performed for 16 h in the presence of 1% DCC-FBS in phenol red-free D-MEM. At 24 h after transfection, cells were treated with vehicle alone, 2 nM EGF, 10 nM estradiol-17β, or 1 µM ICI 182,780. CAT reporter assay was performed after 18 h of treatment using the CAT enzyme-linked immunosorbent assay kit from Roche Molecular Biochemicals (Indianapolis, IN). Equal amounts of protein were analyzed in duplicate for CAT activity, and data were collected from at least three independent experiments.

Cell Proliferation Assay

Proliferation assays were a modification of methods described elsewhere (10,71). In brief, prior to each transfection, COS-7 cells were maintained in phenol red-free D-MEM containing 1% DCC-FBS for 48 h (66). Cells were

transfected in six-well plates using Lipofectamine Plus according to the manufacturer's recommendations (GIBCO-BRL, Life Technologies) (72) at the following concentrations: 4 μ L/well of Lipofectamine; 6 μ L/well of Plus reagent; 1 μ g of pCDNA₃ER-WT, pCDNA₃ER-Y537F, or pCDNA₃ER-Y537A expression plasmid; and 1 μ g of pEV7-HER1 for a total of 2 μ g of DNA per well. Duplicate wells were transfected using 2 μ g of pCMV β gal/well. After 5 h of incubation, the medium was aspirated and new phenol red-free D-MEM containing 5% DCC-FBS was added. After 24 h, each well was divided into 6 wells of a 12-well plate and half were treated with 2 nM EGF in phenol red-free D-MEM, 1% DCC-FBS for 72 h. Cell numbers were determined by direct counts using a hemocytometer. Final data were determined from a minimum of four independent experiments.

Apoptosis Assay

Cell cultures were plated in standard media for 48 h, then changed to analyzed for apoptosis using a detection system described previously (45,74). Apoptosis was assessed by a specific colorimetric detection system (Promega, Madison, WI) (73,74). In brief, fragmented DNA of apoptotic cells were end labeled using a modified TUNEL assay. Biotinylated nucleotide was incorporated at 3'-OH DNA ends using terminal deoxynucleotidyl transferase. Horseradish peroxidase-labeled streptavidin was then bound to biotinylated nucleotides and detected using peroxidase substrate, hydrogen peroxide, and the stable chromogen diaminobenzidine. Using this procedure, apoptotic nuclei stained brown. An apoptotic index was estimated by the percentage of cells scored with a light microscope at $\times 200$ (45).

Statistical Analysis

In each experiment, data are presented as mean \pm SEM. The data in each experimental treatment group were compared with that in the control group using a *t*-test for paired or unpaired observations as appropriate by conventional methods (75), with probability values given in parentheses.

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MEMBRANE-ASSOCIATED ESTROGEN RECEPTORS AND BREAST CANCER

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INTRODUCTION

Membrane-initiated estrogen signaling is characterized by rapid onset of action and by relative insensitivity, at least in early stages, to inhibition of gene transcription or protein synthesis^{1,2}. These acute responses cannot be explained by traditional models of hormone action wherein steroid hormones act only as nuclear transcription factors. The latter nuclear-initiated mechanism requires binding of estradiol to its cognate nuclear receptor, followed by receptor dimerization and binding with specific estrogen-responsive elements (ERE) in DNA, leading to the later regulation of gene transcription and promotion of cell growth. Nonetheless, integration of nuclear- and membrane-initiated signaling by estrogens likely contributes to the full biologic effects of hormone.

Evidence for Membrane-Associated Forms of Estrogen Receptors

The first compelling evidence for membrane-associated estrogen receptors in plasma membranes of target cells was reported in 1977 by use of a ligand affinity-binding approach³ (Table 1). It is now well-established that estrogens interact with specific hormone-binding molecules at the membranes of estrogen-responsive cells⁴⁻⁸, including those of breast cancer cells⁹. Later, using antibodies to ligand-binding and other domains of nuclear ER, Pappas et al.⁵ and independent investigators^{4,7,8,10} were able to detect ER immunoreactivity in plasma membranes of target cells. Similarly, Chambliss et al.¹¹ used three different antibodies to different domains of nuclear ER and detected a 67-kDa band, the molecular size of nuclear ER, in endothelial cell membranes. Such detection of membrane-associated proteins by antibodies directed to nuclear forms of ER suggest that plasma membrane receptors share significant structural homology with intracellular ER. This hypothesis is supported further by studies of Razandi et al.⁷ demonstrating that transfection of cDNA for ER α or for ER β in an ER-negative cell line results in expression of receptors for both ER α and ER β at the plasma membrane as well as in the cell nucleus. Membrane-associated steroid receptors likely account for no more than 10-15% of the total pool of cellular steroid receptors^{3,7,10,12}. Nonetheless, this level of membrane-associated receptors is comparable to that found for many peptide hormone receptors that also initiate signaling cascades at the membrane.

Table 1. Selected reports on membrane-associated estrogen receptors since 1969

Year	Observation*
1969	Acute stimulation of cAMP by estrogen ¹³
1975	Rapid calcium mobilization by estrogen ¹⁴
1977	Specific plasma membrane binding sites for estrogen ³
1979	Membrane estrogen receptors regulate proliferation ¹²
1981	Cell surface immunologic blockade of estrogen action ¹⁵
1984	Estrogen receptors in endometrial plasma membrane ¹⁶
1986	Membrane binding sites for estrogen in breast cancer ⁹
1994	Estrogen action via cAMP signaling pathway ¹⁷
1995	Membrane estrogen receptors by antibody labeling ⁵ Nongenomic effects of estradiol-17 β ¹⁸
1996	Rapid membrane effects of E2 and MAPK signaling ¹⁹
1999	Membrane and nuclear ER α from single transcript ⁷ Membrane ER activates G-protein, IP3, MAPK, cAMP ⁷
2000	ER associates with signaling molecules in caveolae ¹¹ ER interacts with regulatory subunit of PI3-kinase ²⁰
2002	ER cross-talk with growth factor signaling ^{10; 21}

*Only selected references listed here, with >350 citations on membrane ER published in past 30 years. Estradiol-17 β (E2) and estrogen receptor (ER).

The exact nature of the membrane-associated ER remains an active area of investigation. Estrogen-binding proteins of varying molecular size have been partially isolated from plasma membranes of several cell types^{7;22;23}. Recent work has also demonstrated the presence of membrane-associated ER forms in human MCF-7 breast cancer cells by use of both Western immuno-blot and ligand-blot analyses. Plasma membranes were purified from MCF-7 cells using controlled cell homogenization and subcellular fractionation^{3;12;24}. Antibody to the ligand-binding domain (LBD) of nuclear ER α revealed a predominant band at 67-kD in MCF-7 plasma membranes with high-affinity binding capacity for estradiol-17 β (Fig. 1a). Similarly, ligand-blotting of plasma membranes using a specific estradiol-peroxidase probe also revealed a predominant band at 67-kD (Fig. 1b). With both approaches, a secondary membrane protein band also occurred at 46-kD in plasma membranes (Fig. 1a, b), consistent with independent reports by Russell et al.⁸. Of note, additional minor bands were observed at 62-kD and 97-kD, but the significance of these entities remains to be determined².

ER α antisense oligonucleotides reduce membrane estrogen-binding sites

Antisense RNA can bind sense mRNA to block protein synthesis, and use of antisense oligonucleotides has become a common approach to interfere with the activity of specific proteins^{27; 29; 30}. To assess the relation of membrane-associated ER with nuclear ER, a 15-mer antisense oligonucleotide to ER α mRNA was prepared and used to disrupt normal ER expression in MCF-7 cells (Fig. 1c). The 15-mer construct spans the translation start codon for

human ER α mRNA and is similar to those used previously^{27;28;30}. MCF-7 cells were treated *in vitro* with oligonucleotides for 72 hrs, then harvested for assessing ER binding in plasma membrane fractions as before²⁶. The results show that 1 μ M ER antisense reduces specific estrogen-binding capacity in MCF-7 cell plasma membranes to 22 ± 3 % of that found in cells treated with control missense agents²⁸. Thus, this finding is consistent with independent reports demonstrating that membrane and nuclear forms of ER may derive, at least in part, from the same transcript^{4;7;29}.

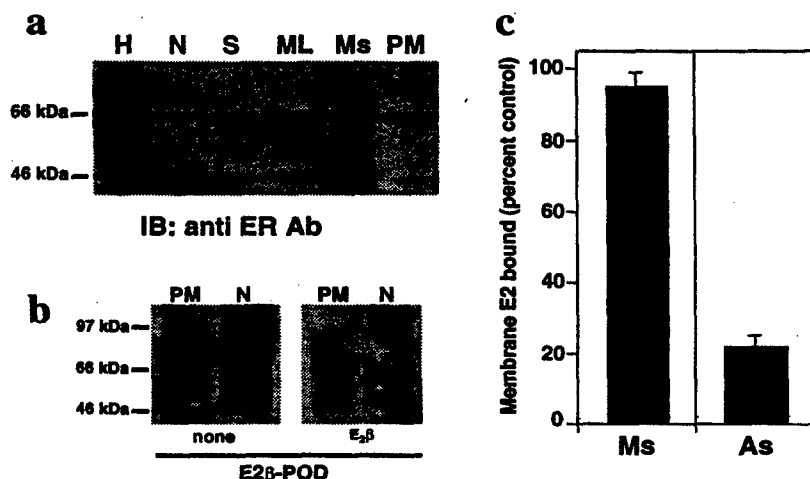


Figure 1. Membrane-associated estrogen receptor forms in human breast cancer cells. Identification of ER in MCF-7 cell fractions by Western blot and ligand-blot analysis: a) Immunoblotting with antibody against LBD of intracellular ER shows presence of a major 67-kDa band in homogenate (H), as well as in nuclear (N), mitochondria-lysosome (ML), microsome (Ms), cytosol (S) and plasma membrane subfractions (PM). b) Ligand-blot analysis with estradiol-peroxidase ($E_2\beta$ -POD) showed binding to a 67-kDa band in plasma membrane (PM) and nuclear (N) fractions. To assess specific binding, $E_2\beta$ -POD binding was tested without (none) and with 10-fold molar excess of free estradiol-17 β ($E_2\beta$)²⁵. c) ER antisense disrupts expression of membrane estrogen-binding activity in MCF-7 cells using established methods²⁶. In brief, oligonucleotides, including a 15-mer ER antisense oligonucleotide, 5'-GGGTCATGGTCATGG-3', and a 15-mer scrambled (missense) control oligonucleotide, 5'-GTGGTGGATCGTGAC-3', were synthesized. Missense oligonucleotide had little to no homology to any known gene sequences. Oligonucleotides were modified as phosphorothioates and purified by NENSORB PREP nucleic acid purification columns before use, with experiments conducted by established methods^{27; 28}.

Biologic Activities of Membrane-Associated Estrogen Receptors

Estrogen plays an important role in the genesis of human breast cancer and in the growth and progression of established tumors. In MCF-7 cells, estradiol enhances the activity of signaling cascades that stimulate mitogen-activated protein kinases (MAPK/ERK)¹⁹ and Akt kinase¹⁰ pathways closely related to control of cell proliferation and survival, respectively. These coordinated actions may contribute to net proliferative effects of estradiol in breast cancers. It is notable that many peptide growth factors activate MAPK through a Shc-mediated mechanism, and studies by Song et al.²¹ now

demonstrate that rapid effects of estrogen in MCF-7 cells occur by activation of classical growth factor signaling pathways that involve ER α as a key mediator. Physical association of ER α with Shc and subsequent formation of Shc-Grb2-Sos complexes appears to be a requirement for phosphorylation of MAPK²¹. The association of membrane ER with other critical cell signaling pathways has also been documented (Table 1).

Antibodies used to demonstrate the presence of membrane ER forms have also helped to reveal potential functional activities of these surface proteins. For example, incubation of MCF-7 cells with antibodies to the ligand-binding domain of ER α led to inhibition of estrogen-induced proliferation of the breast cells¹⁰. This growth-inhibitory effect of anti-ER antibodies correlated with acute suppression of estrogen-induced activation of MAPK and Akt kinase, thus suggesting a correlation between membrane ER signaling and regulation of cell proliferation and survival. Corresponding studies by Norfleet et al.³¹ show inhibition of estrogen-stimulated prolactin release in GH3/B6/F10 pituitary cells by antibody directed against the hinge region of nuclear ER. In addition, Morey et al. report that ER antibody H-222 (against LBD) reversed inhibitory estrogen-induced effects in vascular smooth muscle cells³². Further, Chambliss et al.¹¹ found that inhibition of estradiol-induced eNOS activation in endothelial cells was prevented by prior incubation of plasma membranes from the cells with an anti-ER α antibody.

It remains unclear how membrane-associated ER participates directly in signal transduction cascades initiated at the surface membrane. To date, there is no evidence that ER occurs as a transmembrane protein, or that it is modified to enhance association with the plasma membrane. Nonetheless, one possibility is that membrane ER associates or interacts directly with other membrane-associated proteins that function to modulate signaling pathways (Fig. 2). The enrichment of ER forms in caveolae^{11;33;34} and caveolae-related plasma membrane subfractions supports this hypothesis. Caveolae and caveolae-related structures in plasma membranes, such as lipid rafts, communicate with the cell surface and the interior, and these scaffolding structures are involved in aggregation and integration of diverse signal transduction platforms that regulate growth and survival. It is notable that caveolae occur in most cell types, although caveolae have significantly reduced expression in breast cancer cells³⁵. Caveolae and lipid rafts are also enriched in several growth factor receptors, including members of the EGF receptor family and, as noted above, a portion of ER in target cells co-localizes in caveolae and rafts^{11;33;34}.

Further work is now required to determine whether membrane-associated estrogen receptors are classical forms of ER complexed with other membrane-associated proteins, new isoforms of ER in membranes, known molecules (kinases, ion channels, other receptors) with previously unrecognized binding-sites for steroid, truly novel membrane proteins, or a combination of these^{1;37}

(Fig. 2). It is possible that activation of this alternate signaling pathway by estrogens may represent an important mechanism by which estrogens regulate cell proliferation and survival.

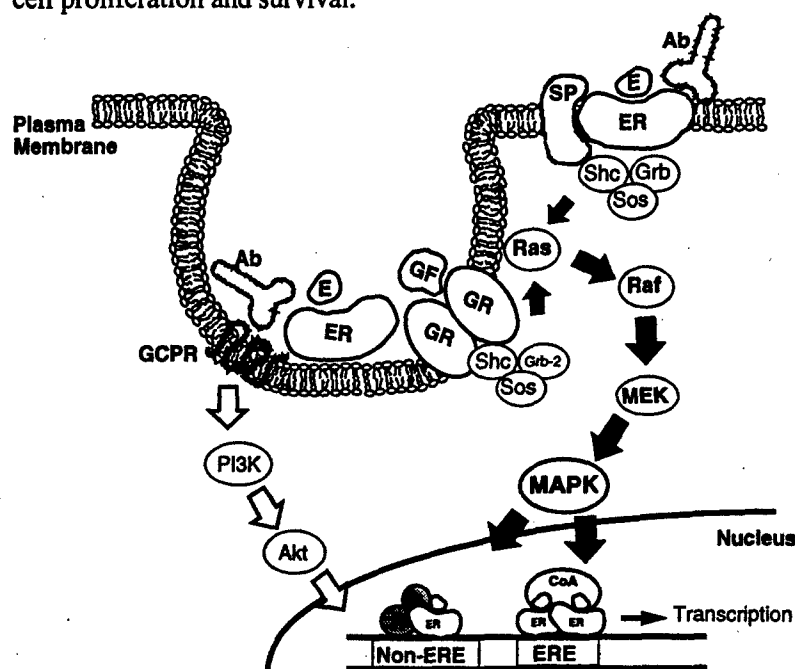


Figure 2. Postulated mechanisms of estrogen action and pathways for cross-communication with other membrane-initiated signaling pathways.

In the most common model of estrogen action, estrogen (E) binding to estrogen receptor (ER) in the nucleus promotes formation of receptor dimers and phosphorylation that favors enhanced binding with coactivator (CoA) proteins and specific estrogen-responsive elements (ERE), leading to selective gene transcription. In some tissues, estrogen may also regulate transcription of genes lacking a functional ERE by interacting with transcription factors such as AP-1 (Non-ERE)³⁶. Nonetheless, pure genomic models do not account for rapid responses to estrogen or early cross-talk with other membrane-initiated signaling networks. Estrogen may also bind membrane ER to promote responses via complementary pathways that cross-talk or interact directly with genomic mechanisms. ER may be recruited to caveolae or associate with membrane via scaffolding proteins (SP). Membrane ER may then affect several pathways, including interaction with transmembrane growth factor receptors (GR); G-proteins (GCPR), nucleotide cyclases, MAP kinase or PI3/Akt kinase (with multiple sites for cross-communication not all depicted here). The fully integrated cell response to estrogen may occur as a result of a synergistic feed-forward circuit where estrogens activate membrane signaling pathways that, in turn, enhance transcriptional activity of ER in the nucleus.

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Steroid and Growth Factor Receptors : Cross-Talk and Clinical Implications

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Summary

Estrogens promote the growth of breast cancer cells by binding with specific receptors. These estrogen receptors are phosphoproteins, and their biologic function can be significantly altered by changes in their phosphorylation state. Signal transduction by growth factor receptors, including epidermal growth factor (EGF) and HER-2/*neu* receptors, can modulate the phosphorylation of estrogen receptor (ER) and the biologic activity of ER-dependent signaling networks both in the presence and in the absence of estrogenic ligands. These molecular interactions can significantly impact breast cancer cell growth and survival. Sensitive and reliable assays of estrogen, HER-2/*neu* and EGF receptors may be important biologic factors to consider in the choice of antitumor therapy for patients with breast cancer.

Keywords: Estrogen receptor, breast cancer, epidermal growth factor, EGF receptor, HER-2/*neu* receptor, c-erbB-2, signal transduction.

Introduction

Growth of normal and malignant human breast tissue is closely regulated by steroid hormones such as estrogens as well as by peptide growth factors that interact with epidermal growth factor and HER-2 receptors^{8,19}. At diagnosis, approximately 75% of human breast cancers are found to be estrogen receptor (ER)-positive, while 25-30% exhibit overexpression of HER-2 receptor, and both the presence of ER⁶¹ and the overexpression of HER-2⁹⁰⁻⁹² are known to be important prognostic factors in human breast cancers. Findings from several studies evaluating HER-2 and ER expression in the same human breast cancer specimens consistently demonstrate an inverse relationship between HER-2 and ER expression levels, with about 50-65% of HER-2-positive samples showing absence of ER^{1,28,109}. Correlation with clinical outcome suggests that tumors with overexpression of HER-2 respond poorly to endocrine therapy with anti-estrogens regardless of ER phenotype^{14,55,105}. These data imply a biologic interaction between ER and HER-2 receptors in human breast cancer, and significant cross-communication between the HER-2 receptor pathway and ER has been reported^{10,28,78}.

The estrogen receptor (ER) is a member of a large family of nuclear receptors that share a common structural and functional organization. These receptors are generally considered to function as ligand-activated transcription factors^{32,46}. However, accumulating evidence shows significant cross-communication between steroid hormone receptors and peptide growth factor signaling pathways, with some reports suggesting that growth factors may promote activation of steroid receptors even in the absence of natural ligand (see Table 1). Agents capable of exerting such ligand-independent activation of ER include epidermal growth factor (EGF)^{17,45,41,42}, TGF- α ⁴³, heregulin⁷⁸, insulin⁷³, IGF-I^{43,45,53,66,95} and dopamine⁸². Under estrogen-free conditions, *in vivo* administration of EGF alone mimics the effects of estrogen in the mouse reproductive tract^{24,65}. In gene knockout mice lacking ER- α expression, both estrogen- and EGF-stimulated uterine growth is blocked²⁴. Thus, ER and coactivator partners may mediate the transcription of target genes by integrating signals from growth factor-activated pathways as well as from steroid hormone binding³³.

Subversion of growth factor receptor function often occurs in malignant progression, with members of the erb B family most frequently implicated in human cancer⁸. The EGF receptor (EGFR/HER-1) is a 170-kD transmembrane glycoprotein that consists of an extracellular ligand-binding domain in its amino-terminus, a transmembrane spanning region and a cytoplasmic EGF-stimulated protein tyrosine kinase in its C-terminus. Upon binding of growth factor with the extracellular domain of its receptor and dimerization, the receptor undergoes autophosphorylation on tyrosine residues. EGFR activation results, in turn, in phosphorylation of downstream protein kinases, such as MAP kinase and PI3 kinase/Akt kinase, and the subsequent activation of specific transcription factors. The EGF receptor family also includes the HER-2/*neu* (erb B2) protein, a 185-kD transmembrane tyrosine kinase encoded by HER-2/*neu* oncogene, the HER-3 protein, a 180-kD membrane receptor tyrosine kinase and HER-4, a 180-kD tyrosine kinase. The HER-2 tyrosine kinase receptor functions in a fashion similar to EGFR¹¹². In addition, upon binding of ligands to EGF, HER-3 or HER-4 receptors, HER-2 receptor is often recruited as a preferred partner of these ligand-bound receptors to form active, phosphorylated heterodimeric complexes that, in turn, activate downstream signaling pathways involved in the growth and survival of tumors. It is notable that cooperative interactions between erb B and erb A (such as estrogen receptor) receptor were first reported more than a decade ago⁹. With emerging evidence for estrogen receptor-stimulated activation of MAP kinase⁴⁵ and PI3 kinase/Akt kinase^{59,88} signaling pathways, growth factor and steroid hormone-dependent mitogenic cascades may well have significant interactions.

Estrogen Receptor Structure and Function

Estrogen receptor- α functions in the nucleus as a transcription factor. The nuclear actions of estrogen are determined by the structure of the ligand, the subtype (ER- α , ER- β) or isoform (transcriptional splice variants, post-translational modifications) of the ER, the characteristics of the gene promoter, and the balance of coactivator and corepressor molecules that modulate the transcriptional response to the estrogen-ER complex³⁶. ER- α is characterized by six major functional domains often termed A-F (Fig. 1). The A/B region contains a N-terminal transactivation domain AF-1; the C region harbors the DNA-binding domain, while the D-region is involved in nuclear localization signaling; and E/F contains the C-terminal portion of the receptor and is involved in hormone-binding, dimerization and the function of a second transactivation domain, AF-2^{46,98}. AF-1 and AF-2 appear to contribute synergistically to the transcription of ER-regulated target genes, but they have different mechanisms of activation.

On binding estradiol, ER- α undergoes an alteration in the conformation of the ligand-binding domain to form a novel surface in the region of the C-terminal helix, helix 12¹⁶. This conformational shift appears to allow the binding of coactivators and other regulatory proteins³⁶. Ligand-bound estrogen receptors function directly as transcription factors by binding DNA as homodimers to specific sequences called estrogen-responsive elements (ERE), generally comprising short palindromic sequences in the vicinity of target genes^{35,102}. Activation of ER ultimately leads to its down-regulation in those cells expressing it. Blockade of this ER signaling pathway by interfering with binding of estrogen to its receptors is the basis of the major hormone

treatment modality, tamoxifen, a partial agonist well-known to limit the proliferative effects of estrogen in the breast. In some tissues, estrogens may also indirectly regulate the transcription of genes that lack functional estrogen-responsive elements by modulating the activity of other transcription factors, such as activating protein-1 (AP-1)^{36,102}.

Ligand-Independent Activation of Estrogen Receptor

Growth factors, such as EGF^{25,34,70}, and estrogens³⁷ are known mitogens for breast cancer cells (Fig. 2), and activation of ER by growth factors in the absence of estrogen is a well-documented phenomenon that may play a critical role in steroid receptor signaling and breast cancer development^{8,24,45,68,78,93}. Several studies document significant interactions between ER and the HER-2 receptor signaling complex (see Table 2). The common inverse association between ER and HER-2 expression in invasive human breast cancers is poorly understood. Earlier studies of interactions between estrogen and HER-2 in breast cancer cells have shown that estrogen can transiently decrease the expression of HER-2 receptor^{84,86}. With regard to the problem of development of hormone resistance, it is notable that some experimental data show that long-term treatment *in vitro* with anti-hormone drugs, including tamoxifen, elicits enhanced expression of HER-2 and EGF receptors in tumor cells⁶⁹. Independent studies suggest that long-term suppression of ER may, in turn, be mediated by HER-2 signaling pathways⁷⁸. In laboratory studies, introduction in breast cancer cells of additional copies of HER-2/*neu* gene with the attendant increase in expression of its protein leads to a significant reduction in the sensitivity of these cells to both estrogen and antiestrogens^{10,78}. ER-positive and HER-2-overexpressing primary breast cancers also show evidence of a deficient antiproliferative response to endocrine therapy using an antiestrogen or an aromatase inhibitor²⁹.

The exact mechanism(s) linking the HER-2 and ER systems, however, are as yet incompletely defined. The estrogen receptor is a phosphoprotein, and phosphorylation of ER occurs early in its activation by ligand binding¹⁰⁰. Some studies suggest that ER phosphorylation at serine and tyrosine residues contributes to receptor activation and, possibly, binding to DNA^{7,46,73,102,108}. The transcriptional activity of AF-2 is activated by binding estrogens, but transcription mediated by the AF-1 domain of ER appears to require phosphorylation of serine-118 by mitogen-activated protein kinase (MAPK) signaling pathways^{2,45}. Thus, growth factor-stimulated activation of ER appears to be regulated, in part, by the AF-1 domain of ER. MAPK-induced phosphorylation of ER may lead to ligand-independent ER activation with loss of the inhibitory effect of tamoxifen on ER-mediated transcription, providing a potential mechanism for the association of growth factor signal transduction with tamoxifen resistance^{17,45,51}. Additional serine phosphorylation sites in ER that may participate in the transcriptional activation of ER include serine-167, a major estradiol-induced phosphorylation site on ER⁷, as well as serine-104 and serine-106⁵⁴.

Although MAP kinase-mediated phosphorylation of serine residues plays a role in the activation of AF-1 in the absence of estrogen, full activation of the AF-1 domain appears to require that other residues, as yet undetermined, must also be phosphorylated⁴⁵. Phosphorylation of ER at tyrosine residues occurs⁶³, and previous data have demonstrated enhanced tyrosine phosphorylation of ER after stimulation of tyrosine kinase signaling in MCF-7 cells by heregulin,

a ligand for HER-1/HER-2/HER-3 receptors⁷⁸. Blockade of estrogen-induced growth of human breast cancer cells by tyrosine kinase inhibitors provides further evidence of the importance of tyrosine kinase pathways in ER signaling⁸⁵. One phosphorylated tyrosine residue in human ER- α , tyrosine-537, is located at the N-terminus of helix 12 in the hormone-binding domain. Although several recent studies show that phosphorylation of estrogen receptor at tyrosine-537 is not an absolute requirement for hormone binding to ER or for activation of ER-dependent transcription^{60,101,102,108,110}, phosphorylation at this site may disrupt hydrophobic interactions that normally maintain the receptor in an inactive state. Thus, it could represent an alternative mechanism for ligand-independent activation of ER, possibly as a consequence of realignment of helix 12 to form an interacting surface for recruitment of coactivators¹⁰². However, identification of a growth factor signaling pathway that phosphorylates and activates the receptor at tyrosine-537 has been elusive. One new study suggests that EGFR tyrosine kinase interacts directly with ER in solution and in intact cells, leading to phosphorylation of ER at tyrosine-537 and tyrosine-43, and these alterations in ER may then contribute to promotion of estrogen-independent activation of ER-mediated transcription and cell proliferation⁶⁰. It remains to be determined what contribution tyrosine phosphorylation may make in regulating the activation of AF-1 or the interactions between AF-1 and AF-2 domains of ER.

Resistance to Hormonal Therapy and Growth Factor Signaling

It is noteworthy that there is precedent for cross-communication between growth factor receptor signaling and the activity of other steroid hormone receptors. In advanced stage breast cancers, progesterone may selectively enhance the sensitivity of key kinase cascades to growth factors, thereby priming cells for stimulation by latent growth signals and allowing a switch from steroid hormone to growth factor dependence⁵². The progression of human prostate cancer from a hormone-sensitive, androgen-dependent stage to a hormone-refractory, androgen-independent tumor may occur, in part, by modulation of androgen receptor signaling by HER-2 tyrosine kinase^{23,107}.

The resistance or insensitivity of some breast tumors to hormonal therapy, such as tamoxifen, may be due, in part, to the activity of growth factor signaling pathways that converge with ER. Although the structural features of ER required for its activation by growth factor signaling are not completely understood, some data suggest that growth factor pathways may target different regions of ER depending on the presence or absence of estrogen, potentially as a result of different conformations of the receptor induced by estrogen^{94,102}. Growth factor signaling appears to stimulate ER transcriptional activity even in the absence of estrogen, albeit to levels significantly less than that of estrogen, and it may also increase the magnitude of target gene expression of ligand-occupied ER. New evidence suggests that estrogen receptor coactivators, such as AIB1, may also serve as substrates and conduits for kinase-mediated growth factor signaling to the estrogen receptor³³. Convergence between growth factor and estrogen signaling pathways may thus elicit a synergistic feed-forward circuit, leading to a stronger or more sustained proliferative response in breast cancer cells. Structural alterations in ER elicited by growth factor receptor signaling may sensitize the steroid receptor to ligand or to coactivator interactions and, thereby, activate biologic responses even at suboptimal levels of

estrogens. In this context, partial agonists, such as tamoxifen, may not provide effective therapy, but tumors may remain sensitive to alternative endocrine treatments. Thus, blockade of estrogen production using aromatase inhibitors may have utility in the treatment of growth factor-overexpressing tumors^{31,57}. Therapies that elicit down-regulation of ER, such as ICI 182,780³⁹ and some aromatase inhibitors¹¹¹, may also be efficacious as alternative antitumor agents.

Another approach to treatment of patients with ER-positive, growth factor receptor-positive tumors may be to simultaneously block both growth factor and ER-dependent signaling pathways. Enhanced anti-proliferative effects in HER-2-overexpressing cells with ER are found by combined treatment with antibody to HER-2 receptor and tamoxifen^{78,103}. Similarly, combination of the anti-HER-2 receptor antibody, Herceptin, with the estrogen receptor downregulator, ICI 182,780, is active in blocking *in vitro* growth of breast cancer cells expressing both HER-2 and estrogen receptors⁵⁰. There may also be considerable potential for use of growth factor-selective tyrosine kinase inhibitors, alone or combined with anti-hormone agents, to treat and possibly prevent endocrine-resistant breast cancer^{51,69}. An autocrine growth factor stimulatory loop involving EGF and HER-2 receptors may be critical to the growth and survival of endocrine-resistant cells^{69,78}. In this context, it is important to note that increased signaling through the EGF receptor pathway also results from overexpression of HER-2 receptor, an important signaling partner for EGF receptor in human breast cancers¹⁰⁴.

Alternative Pathways of Estrogen Action

Although the estrogen receptor is generally considered to function exclusively as a nuclear transcription factor, numerous reports document rapid effects of estradiol that appear to be mediated by a membrane-associated form of ER (see Fig. 2)^{36,56,62,75,76,79,80,99}. These membrane-associated receptors have not yet been isolated in pure form, but several lines of evidence suggest that they may derive from the same transcript as nuclear ER⁵⁶ and play a role in cross-communication with other membrane-initiated signaling pathways. New studies provide evidence for direct interactions between transmembrane tyrosine kinase receptors and ER and suggest that such acute cross-talk between growth factor and estrogen receptors may contribute to modulation of estrogen-induced growth^{22,60}. One potential cellular site for interaction between ER and growth factor receptors may be caveolae, specialized microdomains in plasma membrane. Caveolae are thought to occur in most cell types⁵, although with reduced expression in breast cancer cells⁴⁹. Caveolae are enriched in several growth factor receptors, including members of the EGF receptor family^{5,64}, and a portion of estrogen receptors in target cells also localize in caveolar membrane fractions^{21,48,77,87}. It is clear that further work is now required to determine whether membrane-associated estrogen receptors are classical forms of ER complexed with other membrane-associated proteins, new isoforms of ER in membranes, known molecules (kinases, ion channels, other receptors) with previously unrecognized binding-sites for steroid, truly novel membrane proteins, or a combination of these^{79,80}.

Clinical Significance of Cross-Talk Between Estrogen Receptor and Growth Factor Receptor Pathways

One major problem in breast cancer management is the conversion of estrogen-sensitive to hormone-resistant malignancies after initiation of antiestrogen therapy⁴⁷. The molecular basis for this hormone-independent progression of breast cancer is not clear. However, as noted above, enhanced cross-communication between growth factor receptor pathways and ER during cancer progression could contribute to ER activation in the absence of hormone. This development could then result in a reduced response to antiestrogens⁶⁸. Current findings indicate that HER-2, and possibly EGF receptor, plays a leading role in breast tumor progression^{8,69,90-92}. In patients with breast cancer, prognosis is inversely correlated with overexpression and/or amplification of HER-2 or EGF receptor. In addition, an inverse correlation in the expression of ER and HER-2 receptor or EGF receptor in breast cancers correlates with aggressiveness of the disease and with the response to endocrine treatment^{68,74,78}.

Some recent clinical studies suggest that measurement of EGF and HER-2 receptors in breast tumors may be used to select the most effective endocrine therapy (see Table 3)^{31,83}. Several studies offer evidence that ER- or PR-positive, HER-2-overexpressing tumors are less likely to respond to endocrine therapy, primarily tamoxifen^{13,55,81,105,106}, while other trials have presented contradictory findings (Table 3)^{30,72}. Among those studies demonstrating that traditional hormonal treatments are less able to elicit responses in patients with HER-2-overexpressing as compared with non-overexpressing tumors, there are further differences with regard to the most effective form of alternative therapy^{3,31,57}. A recent meta-analysis of seven clinical studies concluded that metastatic breast cancers overexpressing HER-2 were resistant to tamoxifen (estimated odds ratio of disease progression was 2.46)^{26,40}. However, the relative benefit of adjuvant tamoxifen in early breast cancers with HER-2 overexpression remains controversial.

The difficulty in comparing results from different clinical data sets is likely due to several factors. These studies were essentially all retrospective, and standard methods for the assay of biologic factors were not employed. A wide variety of reagents and technologies are in use to detect HER-2 amplification/overexpression in clinical specimens (immunohistochemistry, fluorescence *in situ* hybridization, ELISA for HER-2 protein in plasma, Southern blot), with differing sensitivity and specificity for each approach⁷⁴. It is well known that HER-2 measurements have been plagued with problems of reproducibility⁴⁴. Similarly, measurements of steroid hormone receptors are not uniformly standardized⁴. ER/PR measurements in routine practice are often not as reliable as required for rational management decisions⁴. Further, the significance of subtypes and isoforms of estrogen receptors and their assay in clinical specimens still remains to be considered³⁶. Moreover, the generally negative correlation between HER-2 and ER expression in breast tumors is sometimes not considered in data analysis. As noted before²⁸, different endpoints associated with different disease settings (for example, metastatic, neoadjuvant) and the combination of endocrine therapy with chemotherapy in the reported studies tends to further compromise interpretation of the clinical data.

Although experimental systems indicate that overexpression of HER-2 leads to tamoxifen resistance or insensitivity in breast cancers bearing estrogen receptor, the data from the clinic are less definitive. A randomized prospective trial assigning patients with reliable HER-2 and ER/PR determinations to endocrine treatment or control groups would be ideal to answer this important question⁴⁰. However, in lieu of the latter approach, retrospective evaluation of a large cohort of

clinical tumor specimens using more reliable and sensitive measures of HER-2 and estrogen receptors could be conducted to evaluate the true utility of HER-2 and estrogen receptors in predicting responsiveness to hormonal therapy and in the choice of different endocrine therapies. Further delineation of these complex pathways in breast cancer cells will, hopefully, lead to the design of novel therapies that combine anti-growth factor signaling strategies with more beneficial anti-hormone measures.

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Table 1. Cross-Communication Between Estrogen Receptor and Growth Factor Receptor Signaling Pathways

Year	Selected Observations
1975	Parallels in membrane-initiated signaling by estrogen and peptide growth factors ^{75,76}
1985	Estrogen-induced growth factors of breast cancer cells ²⁷
1989	Cooperative interactions between erb A and erb B receptors in malignant transformation ⁹
1992	EGF action in uterus involves estrogen receptor ⁴¹
1995	Activation of ER by MAP kinase phosphorylation cascade ⁴⁵
1996	Estrogen receptor knockout mice lack estrogen-like responses to EGF treatment ²⁴
1999	Non-transcriptional action of estradiol on Src/Ras/MAPK pathway triggers DNA synthesis ²⁰
2000	PI3 kinase/Akt kinase regulate estrogen receptor activation ^{59,88}
2001	Hyperactivation of MAPK in EGFR or HER-2-overexpressing cells promotes downregulation of ER, with reversal by inhibition of signaling pathways ⁷¹
2002	Direct interactions between growth factor receptor tyrosine kinases and estrogen receptor- α ⁶⁰

Table 2. Estrogen Receptor and HER-2 Receptor Interactions

Year	Selected Observations*
1986	Tyrosine phosphorylation of estrogen receptor ⁶³
1989	HER-2 overexpression correlates inversely with ER-/PR-phenotype in breast cancers ^{1,91,92,109}
1990	HER-2/ER cross-talk: estrogen receptor downregulates HER-2 ⁸⁴ HER-2 overexpressing breast cancers are tamoxifen-resistant ⁶⁷
1991	Tyrosine kinase inhibitors block estrogen-dependent cancer growth ⁸⁵
1992	Tamoxifen resistance with overexpressed HER-2 <i>in vitro</i> ¹⁰
1995	HER-2-induced phosphorylation and activation of ER ⁷⁸ HER-2/ER cross-talk : HER-2 downregulates estrogen receptor ⁷⁸ HER-2 overexpressing cells with reduced estrogen dependence and tamoxifen sensitivity ⁵⁸
1997	HER-2 antibody enhances antitumor effects of tamoxifen ^{78,103}
1999	Overexpression of steroid receptor coactivator AIB1 correlates with HER-2 overexpression in breast cancer ^{15,33} HER-2 amplification impedes antiproliferative effects of hormonal therapy ²⁹ Inhibition of HER-2 tyrosine kinase and MAPK enhances tamoxifen activity ⁵¹
2002	Resistance to tamoxifen-induced apoptosis associated with ER-HER-2 interaction ²²

*See text for additional details.

Table 3. Endocrine Therapy and the Predictive Value of HER-2 Receptor Expression in Human Breast Cancer*

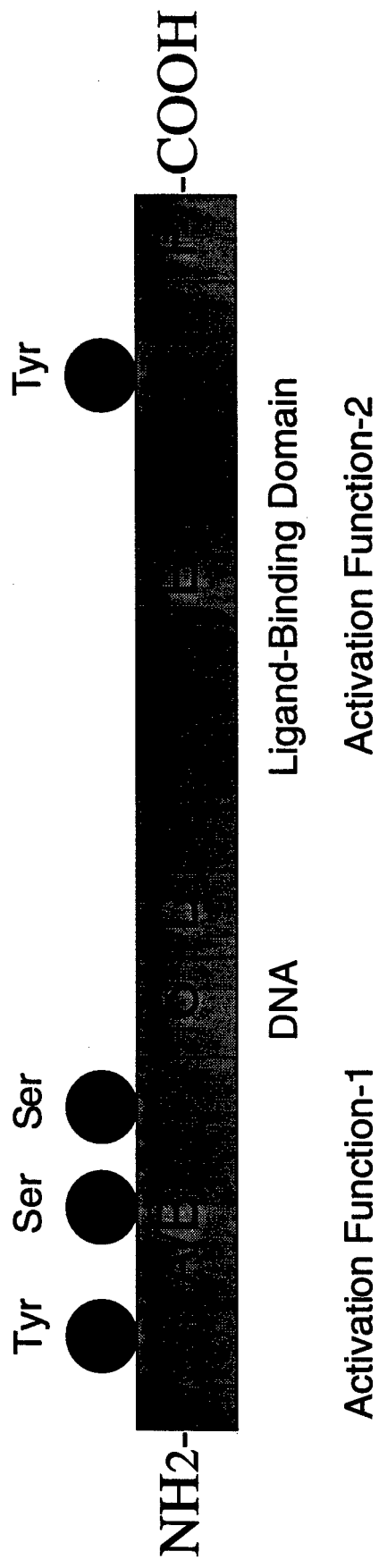
Study	Correlation with HER-2 Overexpression
Wright et al. (1992) ¹⁰⁵	Yes
Borg et al. (1994) ¹⁴	Yes
Tetu and Brisson (1994) ⁹⁷	Yes
Berns et al. (1995) ¹¹	Yes
Leitzel et al. (1995) ⁵⁵	Yes
Archer et al. (1995) ⁶	No
Carlomagno et al. (1996) ¹⁸	Yes
Elledge et al. (1998) ³⁰	No
Sjogren et al. (1998) ⁸⁹	Yes
Houston et al. (1999) ³⁸	Yes
Bianco et al. (2000) ¹³	Yes
Berry et al. (2000) ¹²	No
De Laurentis et al. (2000) ²⁶	Yes
Ellis et al. (2001) ³¹	Yes

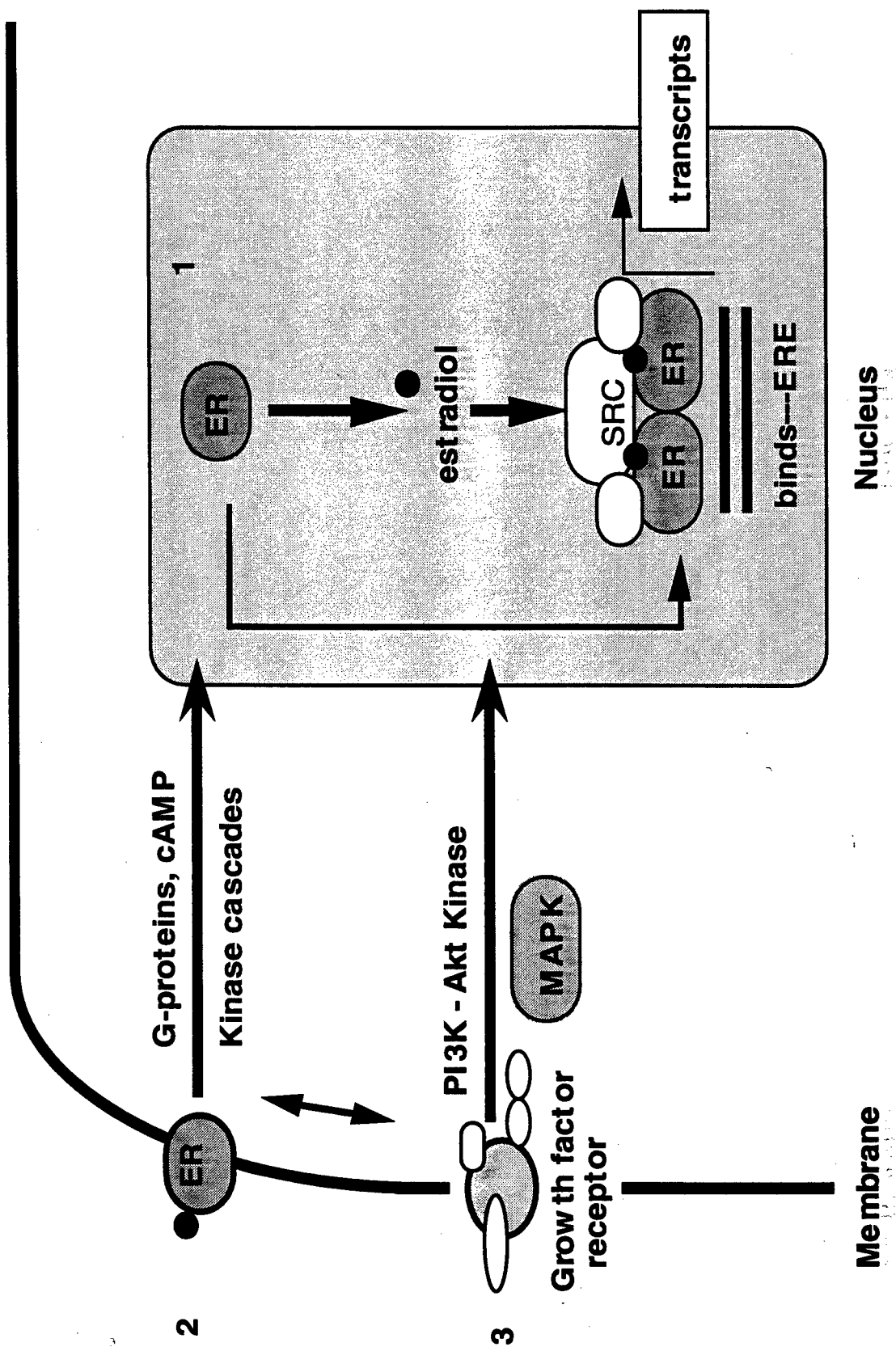
* See text for details and discussion.

Figure Legends

Fig. 1. Functional domains of human estrogen receptor- α . Full-length ER is composed of 595 amino acids. Locations of the DNA-binding domain (domain C) and the ligand-binding domain (domain E) are shown^{94,102}. Positions of the amino-terminal Activation Function 1 and the carboxy-terminal Activation Function 2 transactivation domains are also indicated. The general location of the six known phosphorylation sites in ER- α are represented by the letter P, with notation of serine (Ser) or tyrosine (Tyr) sites.

Fig. 2. Molecular mechanisms of estrogen action and potential pathways for cross-talk with growth factor receptor signaling. In the most common model of steroid hormone action³⁶, estrogen (black circles) binding to estrogen receptor (ER) promotes alterations in receptor conformation favoring enhanced association with coactivator proteins (SRC) and with specific estrogen-responsive elements (ERE) in the nucleus, leading, in turn, to initiation of selective gene transcription (see pathway 1). In some tissues, estrogens may also regulate the transcription of genes lacking a functional ERE by interacting with other transcription factors such as AP-1 (pathway not shown). Nonetheless, the genomic model alone fails to account for rapid cell responses to estrogen treatment or cross-communication with other cell signaling networks^{36,96} (see pathways 2 and 3). The genomic model of hormone action requires integration with alternative pathways of estrogen action. For example, growth factor receptor-induced signaling may interact with ER or other components in the signaling network such as coactivator proteins (pathway 3). In addition, estrogens may bind a membrane-associated ER, with potential for promotion of responses via a complementary pathway that may cross-talk or interact directly with the genomic mechanism (pathway 2). Membrane-associated ER may affect one or more of several pathways, including interaction with transmembrane growth factor receptors; activation of G-proteins, nucleotide cyclases, and/or MAP kinase, with resultant increases in their catalytic products. These interactions may promote phosphorylation of ER itself via steroid-induced or ligand-independent pathways. The biologic responses of breast cells to estrogens may occur as a consequence of a synergistic feed-forward circuit where steroids activate cell membrane signaling pathways that act, in turn, to enhance the transcriptional activity of ER. Active reconsideration of the current genomic model of estrogen receptor action is ongoing, and the probable importance of alternate signaling pathways elicited by membrane binding and cross-communication with other membrane-initiated signaling pathways is now increasingly evident^{56,62,76,80,96,99}.





Plasma Membrane Receptors for Steroid Hormones: Initiation Site of the Cellular Response

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GLOSSARY

Genomic A process related to gene transcription and its regulation.

Nongenomic A process independent of RNA transcription.

Organelle An intracellular, membrane-bounded compartment, *e.g.*, mitochondrion, Golgi, lysosome, endoplasmic reticulum with membrane-bound ribosomes, nucleus, each with specialized functions, reflecting division of labor within cells.

Receptor-mediated endocytosis Cellular entry of **agonist** via a specialized region of the cell where **receptor** molecules, capable of specifically binding hormones, are localized. Such a region may also be rich in specialized proteins, such as **caveolin**. Induced invaginations may be pinched off from the outer membrane and become **endosomes** – vesicular channels for signal transduction.

Signal Transduction A signal is a message relayed from one site to another, in the molecular language of the cell. The *primary* signal, in the hormonal context, originates from binding of the **agonist** (active agent, the hormone) to **receptor** protein at the surface of the **target** cell. Signal transduction involves message conversion (translation) from one molecular "language" to another, "read" elsewhere in the cell – *e.g.*, surface interactions on hormonal impact lead to abruptly altered intracellular levels of substances with catalytic activities, such as Ca^{2+} , cyclic nucleotides, and phosphokinases (which shuttle phosphate between critical proteins, altering their structure and behavior). Thus, signal transduction, like a molecular relay, advances the hormonal message, both temporally and spatially, among the cell organelles, like a lighted fuse, progressing toward output at the terminal.

Steroid A family of lipid structures related to the parent substance, cholesterol, which is modified by enzymes in certain tissues that synthesize highly active products with hormonal functions, such as estrogen and progesterone in ovary, testosterone in testis and cortisol in the adrenal cortex (see Table I).

I. Introduction

It seems axiomatic that mutual recognition between an agonist in the extracellular fluid and the responsive cell must take place at the surface membrane that constitutes the dynamic boundary between them. This fundamental process, first

envisioned in the immunologic context by Paul Ehrlich (Fig. 1), is shared by hormones of varied structure, lipid as well as peptide. This concept is supported by a large body of evidence that has brought into harmony previously divergent views of the significance of the chemical nature of the agonist in the chain of secondary mechanisms that stem from the all-important primary step of selective interception of hormone by receptor. Examples of the lines of evidence and criteria for identifying the selectivity, specificity, and affinity of such interaction for the several steroid classes with specialized protein components of the target cell surface will be presented.

Although the cellular actions of steroid hormones were once postulated to be regulated exclusively by receptors in the cell nucleus, thus permitting selective transcription after ligand binding, this genomic mechanism generally requires hours or days before the effects of hormone exposure are evident. In addition to the latter pathway, steroids also elicit rapid cell responses within seconds of administration. The time course of these acute events lends support to the conclusion that they do not require new gene transcription. Rather, many rapid effects of steroids, termed 'nongenomic', appear to be due to specific recognition of hormone at the cell membrane. Hormone-receptor interactions at the surface membrane can initiate a cascade of signaling events that may regulate many cellular functions, both acute and prolonged.

Subsequent entry of steroid hormone into its target cells can be astonishingly swift and requires special strategies to demonstrate its temporal dissociation from binding proper. In some cases, receptor-mediated entry appears to be closely followed by partition into the several intracellular compartments. The available means of such ultrarapid penetration and dissemination will be outlined, as will the potential significance of sequential translocation in the overall cellular response. Accordingly, the proportion of total receptor, localized at a given moment in any cellular compartment, whether plasmalemmal, cytostructural, or nuclear, reflects the metabolic history of the receptive cell.

Finally, it is the purpose of the present article to survey the transduction mechanisms available to such a cell for amplifying and extending the impact of initial surface perturbation by hormone capture. Through such means of communication of the primary hormonal signal can the resultant structural and functional modulations of the several intracellular compartments, including the nuclear, be coordinated into the totality of the cellular response.

II. Supramolecular Organization of the Surface Membrane and Occurrence of Steroid Receptors

Steroid uptake in cells may occur by passive or facilitated diffusion across the plasma membrane or by one of several endocytotic mechanisms. Biophysical studies demonstrate that most steroid hormones are lipophilic molecules that partition deep within the hydrocarbon core of lipid bilayer membranes, even those devoid of receptor proteins. However, these agonists also appear to enter target cells by a membrane-mediated process that is saturable and temperature-dependent.

Membrane Models : From Fluid Mosaic to Lipid Rafts and Signaling Platforms. To understand the nature of steroid receptor association with cell membranes, it is important to consider current concepts of supramolecular organization of the membrane (Fig. 2). The present view of the lateral organization of plasma membrane constituents has been revised significantly from the original fluid mosaic model, wherein membrane proteins were considered to diffuse freely in a sea of lipid, above a critical temperature of 15° C. With the wide array of molecules known to interact rapidly in receptor signaling, it is difficult to imagine how specific signal transduction could occur if components moved randomly in the lipid bilayer. Rather, new findings suggest the existence of macro- and micro-domains of the membrane that serve to concentrate key signaling molecules for efficient coupling to effectors. The concept of a 'signaling platform' has been advanced to characterize a structure in which many different membrane-associated components are assembled in a coordinated fashion.

Evidence now indicates that plasma membrane microdomains termed 'lipid rafts' arise from the phase behavior of lipid components. In the fluid bilayer of the membrane, different lipid species are asymmetrically distributed over exoplasmic and cytoplasmic leaflets of the membrane. In particular, long, saturated acyl chains of sphingolipids cluster in the presence of cholesterol to form a liquid-ordered phase, resistant to detergent solubilization. Saturated acyl chains of glycosylphosphatidylinositol (GPI)-anchored proteins, as well as transmembrane proteins and certain tyrosine kinases, can also occur within these lipid domains. Raft association may concentrate receptors for interaction with ligands and effectors on either side of the membrane, thus facilitating binding during signaling and suppressing inappropriate crosstalk between otherwise conflicting signal transduction pathways.

Endocytotic Adaptations. Caveolae, literally 'little caves', are more specialized raft microdomains that also concentrate and assemble components of several signal transduction pathways (Fig. 2). These membrane structures can be invaginated, flat (Fig. 3) within the plane of the membrane, detached vesicles, or fused together to form grape-like structures and tubules (Fig. 3). Like lipid rafts, caveolae are rich in cholesterol and sphingolipids, but, unlike rafts, they are lined intracellularly with clusters of caveolin protein, a cholesterol-binding molecule that contributes to membrane lipid organization. The growing list of caveolae-associated molecules constitutes a 'who's who' of cell signaling, including receptor tyrosine kinases, G-protein-coupled receptors, protein kinase C, components of the mitogen-activated protein (MAP) kinase pathway, and endothelial nitric oxide synthase (eNOS). In one such example, sub-populations of estrogen receptors are localized to caveolae in endothelial cells, and, in plasma membrane caveolae isolated from these cells, estradiol directly stimulates its receptors which are coupled to eNOS in a functional signaling module to regulate the local calcium environment and blood vessel contractility.

Clathrin-coated pits are independent membrane invaginations, decorated intracellularly with the protein clathrin. They function in endocytosis of nutrients and certain receptors, such as receptor-mediated uptake of low-density lipoprotein-

cholesterol complexes, and also play an important role in signal transduction. Some agonists may be internalized via either clathrin-coated pits or caveolae, with one pathway apparently providing a default entry mechanism for the other under certain conditions.

Raft-dependent signaling is often coupled with endocytotic uptake mechanisms involving rafts as well as caveolae. Also important in this scheme is the actin cytoskeleton, considered to provide constraints for lateral mobility of lipid microdomains and to function in endocytotic trafficking. Endocytosis itself is a diverse set of processes, which promote internalization of specialized regions of plasma membrane as well as small amounts of extracellular fluid (Fig. 3). The best understood form of endocytosis occurs at clathrin-coated pits and involves clathrin and the dynamin GTPase, which promotes pinching-off of the endocytotic vesicle. Caveolae also play an important role in potocytosis, a mechanism for uptake of small molecules across plasma membrane. Finally, some cell types can internalize larger amounts of fluid by macropinocytosis or particulates by phagocytosis (Fig. 3). In most cells, internalized materials are first delivered to early sorting endosomes, which may mature into or be transferred to late endosomes, and, ultimately, to lysosomes. The potential role of the ubiquitin-proteasome pathway in this process remains to be determined.

Steroid Receptor Variability. The precise nature of the association of steroid receptors with plasma membrane remains elusive, primarily because full structural characterization of these molecules is incomplete. The task of identifying these membrane-associated steroid receptors is made more challenging by the recent detection of multiple transcript variants of classical 'intracellular' steroid receptors, and, in the case of estrogen receptor- α (ER- α), by discovery of a structurally-related estrogen receptor form, termed estrogen receptor- β (ER- β), that is the product of a different gene. Both ER- α and ER- β gene products are expressed in membranes, and both receptors are capable of activating acute and late phases of cellular responses through activation of signal transduction cascades.

Estrogen receptor (ER) from target cell plasma membranes is a protein species with high-affinity, saturable binding specific for estradiol. In addition, antibodies to nuclear ER- α recognize surface sites, suggesting that membrane ER has antigenic homology with nuclear ER. Indeed, recent work reveals that membrane and nuclear ER may be derived from a single transcript. Likewise, properties of membrane glucocorticoid receptors closely resemble those of the intracellular receptor. On the other hand, properties of the aldosterone receptor, as well as those of the plasma membrane receptor for 1,25(OH) $_2$ vitamin D $_3$ suggest that membrane receptors for these steroids may be distinct from their 'classical' intracellular counterparts. Collectively, current findings suggest that membrane receptors for steroid hormones are, in certain instances, transcriptional copies or variants of nuclear receptors and, in other instances, products apparently unrelated to these.

Steroid receptors in membranes may also be contained in multimeric complexes with other transmembrane molecules coupled to specific signaling cascades (Fig. 2). In the case of retinoic acid, binding to known membrane proteins, such as mannose-6-phosphate/IGF-II receptors, may occur. Likewise, progesterone congeners bind with moderate affinity to γ -aminobutyrate type A (GABA_A) receptors that comprise ligand-gated ion channel complexes, and pharmacologic levels of estradiol bind with regulatory subunits of independent ion channels in membranes, thus supporting the view that some effects of steroid hormones, at least at high concentration, may be mediated by known membrane receptors with previously unrecognized steroid-binding sites. Finally, despite subtotal ER- α gene knockout, some rapid actions of estradiol still prevail. As with the mixed steroid hormone-binding protein systems known to occur within cells and in their extracellular fluids, it may well be that multiple forms of receptor proteins for steroids coexist in plasma membranes, thus complicating efforts to isolate and characterize the individual binding species in this cell compartment. Nevertheless, available evidence suggests that a finite portion of cellular steroid receptors is associated with signaling platforms in specialized microdomains of the plasma membrane.

III. Specific Binding of Steroid Hormones to Surface Membranes of Responsive Cells

As postulated by Ehrlich in the Croonian Lecture to the Royal Society more than a century ago, the outer surface of a responsive cell is equipped with specialized components, which exhibit exquisite discriminatory capacity toward potential agonist when molecular conformations are mutually complementary (*cf.* Fig. 1). Indeed, in evolutionary terms, steroid recognition at the surface membrane appears to have been the primary response pathway of the primitive cell. In plant cells, the only known response pathway to steroids is via a membrane-associated receptor that regulates numerous functions in the intracellular economy, including growth and development. In the case of steroid hormones which influence the functions of eukaryotic cells, the fact that such receptor molecules are poised to extract agonist from its plasma protein carrier is directly attributable to primary evidence for *non-covalent*, and thus, reversible, steroid:protein interaction. This property forms the basis for competitive displacement of ligand by excess, or by conformationally-competent congeners.

The concept of specific membrane-associated binding sites for steroid hormones has been supported by rigorously controlled observations from many independent laboratories. Evidence is now available for the extended steroid family, which (Table I) includes the retinoids, thyroid hormone, and digitalis-like steroids (*cf.* Table I). The methodologic approaches have also been [Fig. 4
Fig. 5
Fig. 6] broad. Representative examples of several of these approaches for estrogen are presented in Figures 4-6. However, comparable observations are available for other members of the steroid family, especially adrenocortical steroids and vitamin D metabolites (Table II) (Table II). Thus, from physical, ultrastructural, immunologic and molecular probes, as well as direct kinetic analyses of specific binding of isotopically-labeled steroid to the surfaces of isolated target cells or to their purified plasma membrane

fractions, a large body of evidence now supports this view. Such membrane proteins constitute a fraction of total receptor molecules available at any given moment in the cellular target and have occasionally been overlooked when methods of sufficient sensitivity were not utilized and when signal-to-noise ratio was not taken into account. Especially instructive data are now available for pinpointing the surface orientation of specific receptor proteins for given steroid hormones at their cellular targets (Figs. 4-6). Recent ultrastructural studies have revealed extranuclear immunoreactivity for ER- α associated with membrane sites along dendritic spines and axon terminals of neurons (Fig. 6). Moreover, Fig. 5 reveals incipient receptor-mediated endocytosis in Hep G2 cells. These modern findings confirm the observations of Williams and Baba in 1967, at which time they reported, using electron microscopy and admitted excess of labeled steroids, that [3 H]-aldosterone and [3 H]-cortisol associated with plasma membranes of their respective target cells. It is uncanny that report of abrupt stimulation of membrane-associated adenylate cyclase activity by physiological levels of estrogen appeared in the same year, but these data, as fine red wine, required many years of aging before appealing to the taste of the wider scientific community.

Presently, there has been intensely renewed interest in documenting specific steroid binding with target cell membranes, and current extensions of these data are ongoing. One salient fact emerges from the combined observations, namely, that *there is a striking parallel between the initial encounter of steroid, as well as peptide, agonist with the surface of its responsive cell.* Such function, critical to unfolding of an orderly sequence of succeeding events through receptor-mediated coupling to further metabolic signals (see below), is also shared by many other regulatory agents, including those that promote growth and development of their target cells, such as the phytohemagglutinins in transformation of small lymphocytes, and, indeed, cytokines generally.

It is important to note emerging data, which suggest that different structural conformations of a given steroid hormone may act as specific agonists for selected cellular response pathways. For example, it is suggested that $1\alpha,25(\text{OH})_2$ vitamin D₃ produces biologic responses through two distinct receptors, one predominant in the surface membrane and the other in the cell nucleus, respectively, which are able to recognize different shapes of the conformationally flexible molecule. Accordingly, the functional significance of agonist:receptor interactions at the target cell surface lies in the potential for selective pathway engagement for propagation of this primary signal.

IV. Consequences of Receptor Occupancy: Activation of Signal Transduction Pathways

Repercussions from the cell surface may be communicated to the farthest reaches of cell structure and function, including the transcriptional events that will eventually unfold in the nucleus. Over some decades, manifold activities, which are amplified over the relatively prolonged intervening period, have been documented. In the case of estrogen, which has received the most

attention among the steroid hormones in this regard, the time-course of such events encompasses several orders of magnitude, (Figs. 7-8) leading to its general description as a continuum (Figs. 7 and 8). A similar temporal distribution pattern prevails for responses to glucocorticoids and vitamin D metabolites (Table II).

Propagation of the minimal information from the primary capture of hormone at the cell surface, through an orderly cascade of intermediary reactions in other compartments, and, ultimately, to differentiation or division of the cell so mobilized, begins through recruitment of virtually instantaneous and closely-linked processes within the affected membrane and in its immediate subplasmalemmal environment. Receptor-mediated signal transduction responses have been identified for (Table III) essentially all the steroid hormones (Table III).

An Orderly Cascade. It is significant to note the time-course of these cellular activities, beginning with the earliest indications of membrane perturbation, which occur within seconds or less, such as the nucleotide cyclase reactions. Here, again, is a significant example of mechanism shared by steroid and peptide agonist. It is particularly well illustrated in neural responses. Acute alterations in Ca^{2+} and in $\text{Na}^{+}/\text{K}^{+}$ flux are likewise rapid and occur within wide differences in agonist and end-organ. Abrupt changes in phosphorylation mechanisms, some of which are Ca^{2+} -dependent, are also recruited. Many of these changes in the cytoplasmic microenvironment, in turn, have profound effects upon enzymic reactions and upon cytologic structure, with special reference to protein folding. Thus, amplification of primary hormonal signal is achieved with great conservation of energy and without further input of mass, through a limited number of receptor-mediated transduction mechanisms, linked, in part, through heterotrimeric G-proteins that are integral to plasma membrane. These remarkably conserved features of hormone action are covered in depth elsewhere within these volumes.

In the case of some hormonal responses, interaction at the surface membrane may itself be sufficient to elicit an alteration in cell function. For example, estradiol can directly stimulate protein kinase C activity in membranes isolated from chondrocytes, and the steroid also modulates calcium-dependent eNOS activity associated with its receptor in isolated plasma membranes from endothelial cells. Moreover, estrogens may enhance growth of mammary tumor cells, largely independent of estrogen-responsive element (ERE)-dependent transcription, by stimulating membrane-associated MAP kinase pathways. Ligand-independent activation of steroid hormone receptors also occurs and may represent a more primitive response pathway, whereby cross-communication with peptide signaling systems in the cell can directly modulate the activity of steroid hormone receptors. For example, estrogen receptor can be activated in the absence of estradiol through phosphorylation by EGF-stimulated MAP kinase. Any comprehensive model of steroid hormone action must account for these important cellular interactions.

Transitory Alterations in Cellular Architecture and Translocation of Receptor. Among the numerous, acute responses to estrogen recognition in uterine preparations are brief, transitory alterations in cellular architecture, beyond the clear evidence of regional perturbation (*cf.* Fig. 7), and include incipient vesiculation within the membrane itself (*cf.* Fig. 5). These cytoplasmic responses occur within seconds or less, and comprise striking transitory reduction of arrays of microtubules and microfilaments. Indeed, there is considerable evidence that microtubules and the actin cytoskeleton of the cell play an important role in endocytotic trafficking and concomitant signal transduction. In some cases, such remarkable early modifications of target-cell structure may themselves play a key role in signal propagation by serving to modulate the relative viscosity of the medium in which hormone: receptor [H:R] complex is translocated toward, and into, the nuclear compartment.

The microtubular apparatus, with its arboreal array spanning the sub-plasmalemma and perinuclear/ Golgi regions, has been implicated even more directly in the translocation mechanism for the vitamin D₃ receptor in mouse osteoblasts, as well as in the case of cellular targets to glucocorticoids. There are now clear indications that, at least for some steroid hormones, a significant portion of the hormone:receptor complex occurs in vesicular form (*cf.* Figs. 5-7), with the potential for fusion with other organelles.

Sequential distribution of hormone. Because of the extreme speed of entry, the temporal association of steroid hormone with a surface receptor and its ensuing distribution in target cells has been difficult to demonstrate without appropriate precautions to eliminate nonspecific membrane-perturbing influences. These precautions include strict omission of serum and phenol red from media, use of incubation temperatures at 23° C. rather than the customary 37° C., but not below 15° C., when lipid components of membranes assume a rigid conformation, and, above all, sampling at very short intervals. Indeed, because of lack of appreciation by many investigators of these precautions, cumulative evidence of such association had been overlooked by some for decades.

An especially telling analysis of the [³H]estradiol-17 β translocation mechanism is available, using analytical cell fractionation at progressive time periods, beginning within 10 seconds of exposure. Estradiol-17 β interacts specifically with membrane proteins in uterine cells and undergoes rapid internalization in nanometer-sized endocytotic vesicles resulting in delivery of a portion of the steroid hormone and its associated receptor protein to the cell nucleus and nuclear protein matrix. Quantitative analyses of the postnuclear supernatant prepared from uterine cell homogenates incubated under the strictest estrogen-free conditions indicates that a significant portion of specific estrogen-binding sites is internalized from plasma membranes in vesicular form. Concomitant with a decline in plasmalemmal and presumptive endosomal fractions, a

significant amount of labeled hormone occurs in Golgi and lysosomal compartments before the peak in nuclear accumulation. These observations demand further pursuit with due regard for the scrupulous techniques required.

V. Membrane Signaling and the Cellular Response to Steroid Hormones

Compartmentation in the Cellular Economy. Without some form of communication between the events at the cell surface and the relatively remote nucleus, separated as it is from all else in the cell by a double membrane, the coordinated response of growth or differentiation could not be achieved. Indeed, there is rapidly growing evidence that there is close synergism between the receptor-mediated, virtually instantaneous activities at the plasma membrane and their considerably delayed effects within the nucleus. Clearly, mechanisms exist for transfer of information, as well as matériel, between the two major cell compartments.

Separation of potential reactants by structural barriers of variable degree of penetrability is a primitive yet thermodynamically efficient means of maintaining a poised system. Such a system is capable of rapid responses to changes in its environment if specialized surface components can detect and capture minute amounts of specific regulatory agents. In the fullest sense, the steroid-hormone target cell is just such a system.

The initial stages of the primary response may constitute physicochemical alterations in conformation that promote propagation of signal with the speed of the phase changes reminiscent of the child's game of cat's cradle. The information gap between the cell surface and the boundaries of the other cellular organelles, most notably, the nucleus, is then closed, with variable rates of speed, by a chain of ordered secondary reactions originating from the coupling of liganded receptor to other (Fig. 9) cell signaling proteins (cf. Figs. 8 and 9).

Now, under certain conditions, these transduced responses, in a closely coordinated system of interdependent pathways, forward the expanded signal toward the nucleus and the enhanced genomic activities to come. There have been significant advances in demonstration of hormone and/or receptor in vesicular form, in close perinuclear array at very early times after surface binding and before substantial concentrations occur within the nucleus. The specific means by which the formidable nuclear barrier is crossed have not yet been identified in the hormonal context. However, there are strong indications of organellar intervention and membrane fusion in hormone:receptor complex transport and, in specific cases, delivery through compound lysosomal pathways. At the same time, the ionic, enzymic, and energy-generating functions, recruited in coupled fashion in the cytoplasm, prepare the responsive cell for its expanding metabolic requirements.

The genomic hypothesis of steroid hormone action has generally prevailed as the exclusive mechanism since 1961, the year in which the seminal concepts of Jacob and Monod electrified the scientific community. As will be presented in other

chapters of this Series, in the interval to the present, extraordinary accomplishments by a broad array of molecular biologists have extended and clarified the details of these concepts for the late nuclear repercussions of a number of steroid hormones at their cellular targets, while unfortunately overlooking the well-documented responses attributable to signal at the cell surface. It was inevitable that the emphasis upon the critical and novel activities triggered at the nuclear level would overshadow the parallel observations being made on receptor-mediated signals emanating from the primary recognition site, the cell membrane.

Recent advances have now permitted greater focus upon the acute signals and their systematic transduction. This renewed outlook restores the necessary balance to our understanding of steroid hormone action, and integrates the contribution of each set of functions into a more complete whole (Fig. 9). Moreover, in the case of some hormone responses, the primary interaction at the surface membrane may be sufficient of itself to elicit a cascade of intracellular signals to specifically alter cell function.

Direct, Membrane-Initiated Responses Seemingly Uncoupled from the Cytoplasmic Cascade: The Dual Functions of Surface Receptor Activation. What is not yet clear, except under the special circumstances noted below, is the question of the inexorability of the full sequence of transduction steps from cell surface recognition to genomic activation, and, thereby, to growth or differentiation. Is there a briefer, less extensive pathway—essentially only an abbreviated sequence—that leads to altered cell functions, including those related to the increase in number of osmotically-active particles at a very early stage of structural changes in membrane 'permeability'? As noted briefly above, one such example that comes immediately to mind is the localized liberation of nitric oxide, itself secondary to an instantaneous surge of Ca^{2+} , which occurs in response of endothelia to estrogen; these coupled events result in rapid vasodilation, thus clearly by-passing the hours-long, metabolically expensive transduction pathway leading to nuclear arousal. Such a truncated pathway may parallel only one or two early steps of the full sequential transduction route. The local effects of estrogen on electrophysiological activities of neurons is another obvious case in point. In the instances noted, there is distinct evolutionary advantage to such a short-cut. In fact, there are circumstances currently being identified, in which the two response-sequence stages, full and partial, coexist side by side, thus supporting acute, as well as delayed, responses to surface signal, independently and in parallel.

Accordingly, the functions of the surface receptor are twofold. Both lead to coordination of the activities of more distal organelles. One such function is *complementary* to the more remote and time-delayed events at the genome, through communication of information, both signals and matériel, from the extracellular environment. The second function *supplements* the more delayed and metabolically demanding activities at the genome, through short-cut of the latter. Instead, signals, transduced from receptor engagement of steroid ligand at the external cell surface, are themselves converted, independently of genomic activities, into sharply immediate and readily reversible stimuli, such as those eliciting changes in

nervous activities and vasomotor functions – these, of evolutionary significance for survival. These dual capacities of surface receptor activation underlie perfect adaptation of the receptive cell to the processing of information from its external environment on two independent/interdependent tracks: acute and more prolonged (Fig. 9).

VI. Summary

Rigorously-controlled experimental data, originating from the work of investigators dispersed world-wide, demonstrate that steroid hormones are first intercepted by specialized proteins associated with the surface membrane. Recognition occurs by features of mutual structural conformity, as predicted from principles of physics and chemistry applied to cell biology. For estrogen and glucocorticoid, such receptor molecules at the cell surface have been found to share homology with the nuclear forms, whereas this is not the case for vitamin D metabolites, aldosterone, or thyroid hormone in the limited numbers of tissues examined. Such information is generally lacking for other members of the steroid superfamily.

Capture of steroid agonist from the extracellular fluid is attributable to the competitive advantage of the cellular receptor, for its affinity for ligand is several orders of magnitude higher than that of the carrier proteins in the circulation. This demonstrates that agonist:receptor interaction is reversible, and conforms to the laws of mass action.

Once effective concentrations of hormone are so bound, the cell surface undergoes virtually instantaneous but transitory structural reorganization. These primary interactions may trigger a cascade of specific cellular responses. Thereafter, a portion of the hormone:receptor complex is internalized, generally within seconds or less, through one or more endosomal mechanisms.

Communication and coordination among the several specialized cellular organelles of the targeted cell is achieved by signal transduction processes that propel the hormone:receptor complex or other specific membrane-associated signaling partners toward and into the nucleus. These combined activities are succeeded by the late stages of the response continuum at the genomic level. The outcome is the totality of response in the context of the whole cell, through synergic functions of its organellar constituents.

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We dedicate this work to the memory of our friend and colleague, Dr. James A. Roberts (1946-2001), Professor of Gynecologic Oncology at Stanford University. We thank M. Kowalczyk for expert assistance with illustrations and C. Freeny for technical services in preparation of the manuscript. [Grant support from NIH, NSF, USDA, US Army (DAMD17-99-

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LEGENDS

Figure 1. Diagrammatic representation of the "side-chain" theory to illustrate Ehrlich's concept of specific recognition sites at the cell surface. 1. Complementarity of agonist and receptor. 2. Specific and reversible binding of agonist only to its own receptor. 3. The bound form of receptor is unavailable for providing negative-feedback toward its own biosynthesis. 4. This results in overcorrections by regeneration. Reprinted by permission, with minor paraphrasing of the text, from the Croonian Lecture, "On immunity with special reference to life" delivered by Paul Ehrlich to the Royal Society, 22 March, 1900.

Figure 2. Supramolecular organization of plasma membrane and occurrence of estrogen receptors. A model of the surface membrane from an estrogen-responsive cell in the region of a caveolar structure is depicted. Estradiol may interact with one of several different forms of membrane-associated estrogen receptors (ER). The precise physical and full structural characterization of these molecules remain to be established. They may be known membrane components, such as enzymes, G-proteins, ion channels or receptors for non-steroid ligands, with previously unrecognized binding sites for steroids (1); new isoforms of steroid hormone receptors (2); 'classical' receptors complexed with other membrane-associated proteins (3); or novel membrane proteins (4). Similar to ER, androgen receptor co-localizes with caveolin-rich membrane fractions from target cells, and androgen receptor directly interacts with caveolin-1 in an androgen-dependent process, providing evidence for a potential physiological role of this interaction. Of note, alternatively-spliced transcripts of several steroid receptors occur, and these variant receptors give rise to proteins of different molecular size and, possibly, modified properties. Membrane insertion of receptors in primary transcript form would likely require one or more hydrophobic regions. ER- α , for example, contains several hydrophobic regions, but it is unknown whether these are sufficient for disposition as an integral membrane protein. Post-translational modification of receptor protein leading to cell membrane targeting may also occur, including phosphorylation, glycosylation and/or addition of lipid anchors or other alterations, such as palmitoylation or myristolation.

Figure 3. Schematic representation of pathways for the internalization of extracellular agonists. Revised from Szego and Pietras (1984) and reprinted with permission.

Figure 4. Binding of fluorescein-isothiocyanate (FITC)-labeled antiserum to estradiol and isolated liver cells to estradiol immobilized by covalent linkage to albumin-derivatized nylon fibers. Incubation was conducted at 22° C. with (A) FITC-labeled non-immune serum, or (B) estrogen antiserum, the latter demonstrating availability of the steroid at the fiber surface, as shown in darkfield-UV fluorescence micrographs ($\times 100$). In independent experiments, (C) and (D), cells derived from liver

were incubated with the derivatized fibers in Ca^{2+} , Mg^{2+} -free Ringer solution. Washed fibers with bound cells were photographed with an immersion lens. Some cells appear fairly rounded, while others tend to flatten out at the fiber surface (x850). Reprinted with permission from Pietras and Szego (1979).

Figure 5. Electron microscopic visualization of receptor-mediated, specific binding and internalization of 17β -estradiol-17-hemisuccinate:BSA that had been adsorbed to colloidal gold (E17 BSA: Au) at surfaces of human hepatoblastoma (Hep G2) cells. Note binding of ligand to the plasma membrane directly over a potential clathrin-coated endocytotic pit (arrow head) and intracellular tubulovesicular structures beneath it (small arrows). In control preparations with BSA: Au (lacking derivatization with estrogen; not shown), there is minimal internalization, despite its presence in abundant extracellular concentrations. Scale bar, 0.250 μm . Reprinted with permission from Moats and Ramirez (2000).

Figure 6. Electron microscopic demonstration of localization of immunoreactivity to peroxidase-labeled receptor for alpha isoform of estrogen receptor ($\text{ER}\alpha$) in the hippocampal formation of proestrous rats. Both genomic and nongenomic functions are implicit in the distribution of immunoreactivity. A. Label is seen throughout the nucleus (N) of a neuron in the hilus of the dentate gyrus, as well as a few patches in the cytoplasm (arrowhead), and also at the plasmalemma (small arrows). B. In another cell, a dense patch of immunoreactivity is seen in the nuclear envelope, while in C, an intensely labeled endosome (En) occurs in the perinuclear cytoplasm near the Golgi apparatus (G). Additional $\text{ER}\alpha$ labeling was affiliated with the perikaryal plasmalemma and is apparent in dense patches of reaction product adjacent to several cytoplasmic organelles (B and C). Extranuclear sites revealed with the present methods had not been identified previously by light microscopy. Scale bars = 0.5 μm . Reprinted with permission from Milner *et al.* (2001).

Figure 7. Low-magnification electron micrographic views of luminal surfaces of uterine epithelial cells of ovariectomized rats at brief intervals after iv administration of control vehicle (A) or $\text{E}_2\beta$, 0.5 $\mu\text{g}/100$ g body wt (B-I). Relative paucity of microvilli (MV) in control preparation is in contrast to striking onset and progressive enhancement of these structures at 35 (B), 45 (C), 80 (D) and 120 (E) sec after exposure to hormone *in vivo*. F-I: cell surfaces at 5, 10, 15, and 30 min, sequentially, reveal the remarkable subsidence of the above MV activity. Thus, by 30 min after estrogen (I), the degree of luminal surface investment with MV closely resembles the relatively quiescent control state (A). ds, Desmosomes. Reprinted by permission from Szego *et al.*, (1988).

Figure 8. Schematic representation of time course of responses of uterus to estradiol-17 β . Times shown on the logarithmic scale refer to onset of unequivocal change from baseline values. Thus, times indicated are dependent in part upon sensitivities of the various analytic methods applied and upon the somewhat arbitrary selection of initial time-points for observation in the several experimental protocols. Reprinted with permission from Szego and Pietras (1984), and amplified with further data.

Figure 9. The response as continuum: signal transduction mechanisms leading to the full sequence of receptor-mediated responses of the target cell to steroid hormone. Postulated mechanism of action of a steroid hormone (black circles) in target cells with a steroid hormone receptor (HR) is shown. Steroid ligands bind first to membrane-associated receptors (*cf.* Fig. 2). The liganded membrane receptor may affect one or more of several pathways, including phospholipase C (PLC) or protein kinase C (PKC) signaling, leading to modulation of ion channels and enhanced flux of ions, notably Ca⁺⁺; interaction with peptide or growth factor membrane receptors (GFR) and their immediate signaling partners (SOS, Grb, Ras); activation of MAP kinase cascades (Raf-MEK-MAPK) or G-proteins and nucleotide cyclases (AC) with generation of cyclic nucleotides (cAMP) and modulation of protein kinases (PKA). These primary membrane interactions may promote physical alteration of the steroid receptor itself, such as phosphorylation, via steroid-induced or ligand-independent pathways. In some cases, steroid receptors then associate with vesicular structures and microtubule-microfilament (mf) elements in the cell interior and gain access to other subcellular compartments. Liganded steroid receptor in the nucleus may promote association of the receptor with coactivator proteins and with specific hormone-responsive elements (HRE) in DNA, leading, in turn, to initiation of selective gene transcription. The wide array of cell responses to steroid hormones may occur as a consequence of synergistic feed-forward circuits where steroids activate cell membrane signaling pathways that act, in turn, to enhance the transcriptional activity of specific receptors in the nucleus.

TABLE I

General, Receptor-Mediated Functions of the Steroid Hormone Super-Family

Estrogen	Growth and development of reproductive targets, including breast, bone, liver and cardiovascular system
Androgen	Reproductive tract functions, patterns of hair growth, influences on brain and libido in both sexes
Progesterone	Components of reproductive function and behavior, meiosis in oocytes, acrosome reaction in sperm
Glucocorticoids	Maintenance of integrity of cell membranes; metabolic functions in protein mobilization and gluconeogenesis; neurone signaling; immune and inflammatory reactions; apoptosis
Aldosterone	Promotion of reabsorption of sodium and excretion of potassium in kidney, colon, urinary bladder; acute effects on cardiac function and on sodium transport in smooth muscle
Digitalis-like	Inotropic and chronotropic effects on heart; inhibition of Na^+, K^+ -ATPase in this and many other tissues
Vitamin D	Regulation of Ca^{2+} and phosphate homeostasis; promotion of differentiation of many cell types
Retinoids	Control of cell growth during embryonic development; anti-oxidant function promotes integrity of epithelial and many other tissues
Thyroid hormone	Energy expenditure; embryonic development and postnatal maturation of various tissues, including bone and brain

TABLE II

Parallels in Membrane-Initiated Phenomena Induced by Glucocorticoids and Vitamin D Metabolites

TIME	GLUCOCORTICIDS	1,25(OH) ₂ D ₃
Seconds	Binding to surface receptor Electrophysiological effects Binding to intracellular receptors	Binding to surface receptor Ca ²⁺ channel activation Membrane receptor internalization
Minutes	PKC activation/translocation ¹ Capping of membrane receptors ² Decreased P _i uptake ¹	PKC activation/translocation PKA activation Vesicular loading of P _i , Ca ²⁺ Increased P _i Ca ²⁺
transport ³		Secretion of calbindin, cathepsin B ³ Phosphorylation of osteopontin ⁴
Hours	Enzyme synthesis ⁵ Apoptosis ²	Synthesis of Ca ²⁺ binding proteins Synthesis of α -tubulin ³ Proliferation of lysosomes ³ Cell differentiation/migration

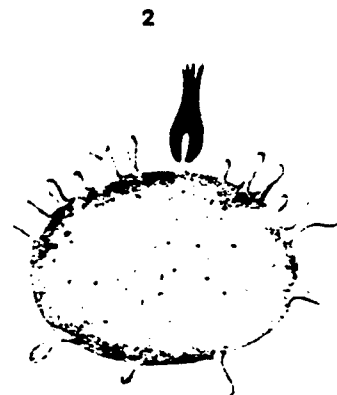
¹Kidney; ²lymphocytes; ³intestine; ⁴bone; ⁵liver

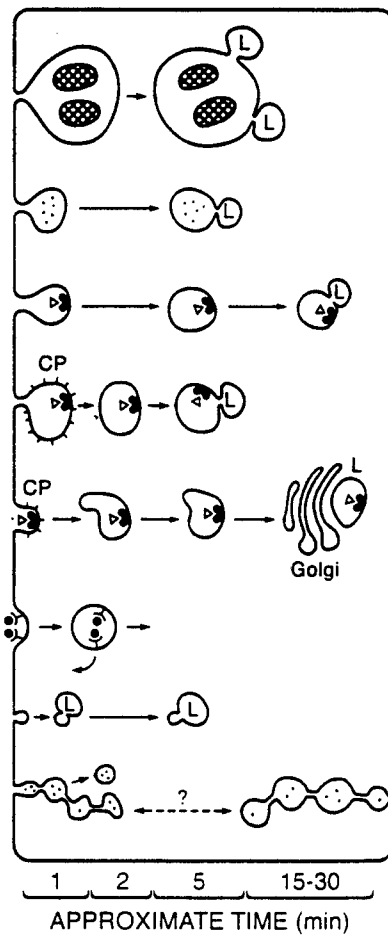
TABLE III

Examples of Acute, Receptor-Mediated Signals of Plasma Membrane Perturbation*

-
- Alterations in Na^+ , K^+ -ATPase activity
 - Rapid shifts in availability of cyclic nucleotides
 - Fluxes in Ca^{2+} and other ions, with potential for modulation of neural activities and numerous enzymatic and mechanoeffector systems
 - Activation of the phosphoinositide cascade
 - Release of endogenous amines and nitric oxide, with influence on microcirculation
 - Structural reorganization of the cell surface, with potential for intracytoplasmic communication; formation of endosomes
 - Accentuated delivery, in microquanta, of components of lysosomes to the cell surface and interior

* Properties shared, to various degrees, by steroid and peptide hormones, as well as many other effectors, including neurotransmitters, lectins, and toxins





PHAGOCYTOSIS: VACUOLES.
dia >1 μ m [Particles]

MACROPINOCYTOSIS: VESICLES.
300-1000nm [Ferritin]

RECEPTOR-MEDIATED MACRO-
PINOCYTOSIS: [HLA-Antigen]

RECEPTOR-MEDIATED PINOCYTOSIS
VIA COATED PIT: 50-250nm
[LDL-cholesterol]

RECEPTOR-MEDIATED PINOCYTOSIS
VIA COATED PIT & 'RECEPTOSOME':
150-350nm [α_2 -MG]

RECEPTOR-MEDIATED ENDOCYTOSIS
VIA CAVEOLAE: 50-100nm

MICROPINOCYTOSIS: 70nm

RACEMOSE VESICULATION:
50-200nm: DIFFUSION

